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MENINGOCOCCAL BROTH CULTURE FILTRATES: FAILURE OF PROTECTION EXPERIMENTS.

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THE poisonous nature of cell-free filtrates of broth cultures of meningococci and other *Neisseriæ* has been demonstrated by many authors (Erlich and others, 1925; Herrold, 1927; Ferry, 1931; Krestownikowa and others, 1933; Maegraith, 1935; Branham and others, 1936), the toxic substances being present in the filtrates within three days of the beginning of growth. Ferry, Norton and Steele (1931), Ferry (1932*a*, *b*, 1934) and Ferry and Schornack (1934) have attempted to show that these toxic substances in the filtrates are antigenic, and give rise to protective antibodies on injection into animals. Although there is some clinical evidence that sera made in this way may be of therapeutic value in cerebrospinal fever (Hoyne, 1936), animal experiments do not appear to confirm the claims of Ferry and his associates. The experiments of Krestownikowa, Belkina, Dosser and Lasowsky (1933), who obtained some protection against the toxic filtrates with antimeningococcal sera, are possible exceptions. Kirkbridge and Cohen (1937) state that marked antitoxic potency against the filtrates could not be shown in either so-called "antitoxic" sera prepared from the filtrates, or in "antibacterial" sera prepared from meningococcal cells. These results are similar to those obtained by Branham, Pabst and Lillie (1936), who came to the conclusion that "antitoxic" sera would protect against neither cultures of meningococci nor toxic filtrates of broth cultures in rabbits or guinea-pigs. Petrie (1937) has reported similar failures with "antitoxic" sera.

The experiments described in this paper (referred to at the International Congress for Microbiology, 1936) have also ended in complete failure to detect protective antibodies in sera prepared from toxic filtrates. If the poisonous properties of the filtrates resulted from the presence of soluble antigenic toxins

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produced by the growth of the organism, then antibodies made from the filtrates should protect animals against their lethal effects. No serum tried in this laboratory has yet been shown to possess any such power of neutralizing the poisonous filtrates.

In a previous communication (Maegraith, 1935) it was shown that the filtrates killed guinea-pigs with regularity only when injected into the cerebro-spinal spaces, intraperitoneal and intravenous injection of even very large doses (up to 15 c.c.) being, as a rule, non-lethal. In the experiments outlined below, therefore, the investigation of the protective action of antisera was mainly confined to the examination of the effects of sera following the injection of the toxic filtrates into the cisterna magna or directly into the subdural space by the method described elsewhere. The protective powers of the various sera against lethal doses of living organisms were not investigated, as it is impossible to ascribe any favourable results gained this way to antitoxic rather than antibacterial properties of the sera. Because of this, most of Ferry's early work on animals is of little value, as he used his "antitoxic" sera to protect against intrathecal inoculation of lethal doses of living meningococci. None of the sera examined exhibited any power of neutralizing skin reactions produced by intradermal injection of toxic filtrates into humans. This technique of examining the sera (also used extensively by Ferry) was not exploited, since the agent responsible for skin-reactions is not necessarily that which kills the experimental animal on subdural inoculation, nor would the power of a serum to protect against a skin-reaction always indicate its power to protect against the poisonous filtrate after subdural injection.

EXPERIMENTAL.

Filtrates.—Toxic filtrates were obtained by filtering through a Seitz filter 4- to 6-day-old cultures of meningococci (usually Group I–III) grown in the blood broth described in a previous paper (Maegraith, 1935). The filtrates were freshly prepared for each experiment and were tested for sterility before use.

Method of testing antitoxic powers of sera.—The various sera used were mixed with the filtrates before injection and incubated at 37° C. for 2 hours. Any precipitate which formed was centrifuged off before use. The sera were mixed in this way with the filtrates, because it was found that their injection either at the same time as the filtrate or before it made no difference to the result. Filtrates and mixtures of filtrate and serum were injected through the skull or into the cisterna magna of guinea-pigs as described elsewhere (Maegraith, 1935). The amounts injected varied from 0·2 to 0·5 c.c., depending on the size of the animal and the toxicity of the particular filtrate. Control injections of the same amounts of blood froth were found to be relatively harmless.

After injection the animals were kept in a warm room for 48 hours and the number of deaths within 24 hours noted. In most cases death occurred well within this time, the average being about 18 hours.

The experiments performed in this way can be divided into two groups: (*a*), the reactions of various antitoxic sera with toxic filtrates, and (*b*), the reactions of antibacterial sera and other substances.

A. *The Reactions of Various Antitoxic Sera with Toxic Filtrates.*

The following antitoxic sera were used in these experiments: Samples of "antitoxic" serum prepared by Parke Davis after Ferry's method; horse-serum prepared by subcutaneous injection of toxic filtrates *plus* intravenous injection of meningococcal cells; and rabbit sera prepared by subcutaneous injection of toxic filtrates. The horse-sera other than that obtained from Parke Davis were prepared by Dr. G. F. Petrie at the Lister Institute, Elstree (Petrie, 1937).

Under the experimental conditions described above none of these sera exhibited any protective properties against subdural injection of toxic filtrates, although in many cases the filtrates were obtained from cultures of the organism from which the antisera had been prepared (usually Robinson, Group I–III).

Examples of the results of some of these experiments are given in Table I:

TABLE I.—*Effects of Subdural Injection of Mixtures of "Antitoxin" and Filtrate.*

Experiment.	Antitoxin.	Mixture. Toxin : antitoxin.	Dose in c.c.	Animals injected. Number.	Animals injected. Weight in g.	Number of deaths.
1	Ferry	4 : 1·5	0·4	7	190–250	7
	,,	1 : 1	0·5	10	600–1000	10
	,,	2 : 3	0·5	4	,,	4
	,,	1 : 1	0·3	5	300–450	5
	Broth only	—	0·4	4	270	0
	,,	—	0·25	4	200–230	0
2	Ferry	1 : 1	0·3	3	435–500	1
	Petrie M. (6 weeks)	1 : 1	0·3	5	190–500	5
	Petrie F. (6 weeks)	1 : 1	0·3	5	200–450	3
	Broth only	—	0·4	4	270	0
4	Petrie F. (6 months)	1 : 1	0·3	10	300–400	8
	Broth only	—	0·3	8	290–310	0
5	Petrie M.	1 : 1	0·3	19	210–360	17
	Petrie F.	1 : 1	0·3	18	,,	16
	Filtrate *plus* normal horse-serum	1 : 1	,,	20	,,	17
	Broth only	—	,,	10	,,	2

Note.—Petrie M. = Horse-serum prepared by Dr. Petrie by injection of both filtrate and meningococcal cells.

Petrie F. = Horse-serum prepared by Dr. Petrie by injection of filtrates only.

In Experiment 2 these sera were taken after 6 weeks' immunization of the horses, and in Experiments 4 and 5 after 6 months.

Ferry = Parke Davis antitoxin prepared after Ferry's method.

All mixtures of filtrate and serum were incubated for 2 hours at 37° C. before injection.

Other experiments in which both Ferry's serum and the sera prepared by Dr. Petrie were tested against various poisonous meningococcal broth filtrates similarly failed to show any neutralizing properties of the sera or gave equivocal results, as, for example, Experiment 3, in which the results were as follows:

Of 7 animals injected with 0·3 c.c. filtrate + Petrie antitoxin 1 : 3, 2 died.
" 8 " " " " " + Petrie antitoxin 1 : 3 + antibacterial serum, 3 died.
" 3 " " " " " + Ferry antitoxin + antibacterial serum, 1 died.
" 6 " " " " " + broth, 3 died.

In this case the filtrate was too weak for the amount injected to kill all the control animals, and experiments performed soon afterwards with fresh filtrate and the same batches of sera showed no protection of animals with any of them.

B. *The Reactions of Antibacterial Sera and Other Substances.*

As in the case of the " antitoxic " sera no antibacterial serum was found to have any neutralizing effect on injections of lethal doses of toxic meningococcal filtrates, although the sera used were all bought in the open market as therapeutic anti-meningococcal sera. Further, the serum prepared by injection of horses with both filtrate and meningococcal cells also failed to protect against the filtrates (see Table I, Exp. 5). The failure of a similar serum has since been reported by Kirkbridge and Cohen (1937).

The antibacterial properties of the sera tried in these experiments were not investigated, as has been done by Kirkbridge and Cohen (1937), who have shown that active antibacterial sera will not neutralize toxic filtrates.

The agglutination titre of the antibacterial sera was usually high, and uniformly higher than that of the " antitoxic " sera. The same was true of the

TABLE II.—*Effects of Subdural Injection of Mixtures of Antibacterial Sera and Filtrates.*

Experiment.	Antiserum.	Mixture Toxin : serum.	Dose in c.c.	Animals injected. Number.	Animals injected. Weight in g.	Number of deaths.
1	P.D.	4 : 1·5	0·4	7	190–260	6
	"	1 : 1	0·5	10	600–1000	10
	Filtrate *plus* broth	1 : 1	0·4	7	200–230	6
	Broth only	—	0·4	4	270	0
3	B.W. 1	1 : 3	0·3	7	600–800	4
	Filtrate *plus* broth	1 : 3	0·3	6	400–800	3
4	B.W. 2	1 : 1	0·3	10	160–360	9
	Broth only	—	0·3	8	290–310	0

Note.—P.D. = Therapeutic antimeningococcal serum prepared by Parke Davis.
B.W. 1 and 2 = Samples of therapeutic antimeningococcal sera prepared by Burroughs Wellcome & Co.

precipitin titre. Thus the mixed antitoxic and antibacterial serum prepared by Dr. Petrie reached an agglutination titre of up to 1 in 2000 and a precipitin titre of 1 in 10^5 (tested against a solution of Group I–III polysaccharide), while the antitoxic serum prepared at the same time had an agglutination titre of only 1 in 250 and a precipitin titre of about 1 in 10^3.

The results of typical experiments with antibacterial sera are set out in Table II.

Addition of normal horse-serum to the filtrate before injection had no effect on the toxicity, and the injection of normal horse-serum alone proved to be non-toxic.

Each fresh batch of blood broth prepared was tested by injection before the meningococci were grown in it, and of such control injections of broth (40 animals) only 2 animals died, one immediately after injection (see Table I).

Attempts were made to adsorb the poisonous substance in the filtrate on to finely-minced guinea-pig brain, but were unsuccessful, and it was also found that neither antitoxic nor antibacterial sera would protect animals against injections of solutions of the toxin precipitated from the filtrates by acetone (Maegraith, 1935).

SUMMARY.

The experiments outlined above demonstrate that the "antitoxic" antibacterial and normal sera examined have no power of neutralizing the poisonous filtrates of broth cultures of meningococci. Sera prepared by injection of these filtrates into rabbits and horses have low agglutinating and precipitating properties, as well as being devoid of protective action against the filtrates.

These results are in agreement with those obtained by other workers referred to above, so that there would appear to be no reliable experimental evidence at present in support of the use of such toxic filtrates for the preparation of therapeutic sera.

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TUMOURS IN RATS AND MICE FOLLOWING THE INJECTION OF THOROTRAST.

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THOROTRAST is a colloidal solution of thorium dioxide containing 19 to 20 per cent. thorium dioxide by weight, and is extensively used in radiology as a radio-opaque substance. Oberling and Guérin (1933), following an investigation on its therapeutic effect on the Jensen rat sarcoma, found large intraperitoneal sarcomata in rats which had absorbed their original transplanted tumours and had lived 10 to 13 months after the intraperitoneal inoculation of thorotrast. In view of the long latent period, the histological appearances of the intraperitoneal tumours and the high degree of immunity which rats acquire to the Jensen rat sarcoma after the absorption of that tumour, they concluded that the intraperitoneal growths were new tumours induced by the thorotrast, and not recurrences of the transplanted tumour. This conclusion was confirmed later by Roussy, Oberling and Guérin (1934), who showed that sarcomata could be produced with thorotrast in normal rats by both subcutaneous and intraperitoneal inoculation. The dose of thorotrast used in their experiments was 5·0 c.c. per rat.

The present investigation was undertaken to find out whether thorotrast, in much smaller doses, would produce tumours in both rats and mice. Besides being of theoretical interest, it was considered worth while confirming and extending the results of the French workers, because of the extensive use of thorotrast in radiology (Selbie, 1936).

EXPERIMENTS.

60 rats were inoculated subcutaneously in the right flank with 0·3 c.c. thorotrast (Heyden, Dresden). A fortnight later a second dose of 0·3 c.c. was given at the same site.

60 mice were given two doses of 0·1 c.c. in the same manner and at the same interval.

The total dose of thorotrast was therefore 0·6 c.c. in each rat and 0·2 c.c. in each mouse.

The injected thorotrast at first formed a rather soft abscess-like swelling, which became firm and nodular after 2 or 3 weeks and eventually formed

hard fibrous nodules, with induration of the surrounding tissues. The thorotrast deposits were palpated at fortnightly intervals, and the development of a tumour could be observed by detecting an increase in the size of one of the nodules.

RESULTS.

Tumours in Rats.

Incidence.—The time of appearance of the tumours is shown in Table I.

TABLE I.—*Tumour Rats.*

Number.	Tumour noted (weeks).	Result of auto-plasts.	Result of trans-plantation.
1	48	..	+
2	54	..	..
3	54	..	—
4	54	..	..
5	54	..	..
6	57	+	+
7	57	..	—
8	57	..	..
9	57	..	..
10	59	+	+
11	59	..	+
12	61	+	+
13	62	+	+
14	62	+	+
15	66	+	+
16	67	+	..
17	67	+	—
18	67	+	+
19	67	+	..
20	68	+	—
21	68	—	..
22	72	..	..
23	73	..	..
24	73	..	..
25	73	..	..

+ = positive.
— = negative.
Blank = not done or death of inoculated animal within 10 days.

52 weeks after the first inoculation there were 43 surviving rats, including the first tumour rat. During the next 10 weeks (to the 62nd week) tumours were observed in 13 rats, and from the 66th week to the 68th week tumours were observed in 7 rats. Meanwhile 4 rats had died without tumours. The remaining 18 rats were killed at 72 and 73 weeks after inoculation in order to find early stages of tumour-formation. In 4 cases small well-developed

tumours were found, giving a total of 25 rats with tumours at the site of the thorotrast injection. This gives a yield of 58 per cent. tumour rats in the 43 rats that lived long enough to have tumours (52 weeks). If the remaining rats had been allowed to live longer there is no doubt that there would have been more tumours. As it was, an early fibroma and an early sarcoma were found in 2 rats, to which reference will be made later. These 2 rats are not included in the tumour rats.

Description.—22 of the rat tumours are spindle-cell sarcomata, presenting various degrees of differentiation. Most of them are poorly differentiated, but in 4 (Nos. 2, 13, 15 and 20) there is a considerable development of collagen, sufficient to warrant the description of fibrosarcoma. In 19 of these tumours malignancy can be established histologically by finding invasion of the *panniculus carnosus*. As the thorotrast granuloma lies immediately under this muscle a sarcoma rapidly infiltrates it, and thus allows a diagnosis of malignancy to be made at an early stage. In one early rat tumour (No. 22) where there is no evidence of invasion of muscle there is direct extension into a lymphatic gland. In tumour rat 7 the tumour is so far ulcerated that the relation of the tumour to the *panniculus* is obliterated; in tumour rat 23, on the other hand, the tumour is so small that no important structure has been involved. In both cases, however, malignancy can be determined by the histological appearances of the tumour-cells.

Glandular metastases were produced in 2 rats. In tumour rat 6 a metastasis could be palpated in the right axilla 1 week after operation. This grew to a considerable size, and when the animal was killed a second small metastasis was found in the right axilla. In tumour rat 14 a small glandular metastasis was found at autopsy in the right axilla.

In the other 3 tumour rats the tumours are fibromata. One is an encapsulated cellular fibroma (No. 4), and another is a fibroma consisting of interlacing bundles of fully developed fibroblasts (No. 11). In the third, No. 21, there are two fibromata, one at either end of the thorotrast deposit. In the centre of one of these tumours there is a deposit of thorotrast, mostly contained in macrophages, and, in close association with this, an area consisting of bands of spindle-cells with no collagenous differentiation and with hyperchromatic nuclei (Fig. 1). Mitoses in this area are numerous, whereas in the main tumour they are few. The characters of this area are so different from the rest of the

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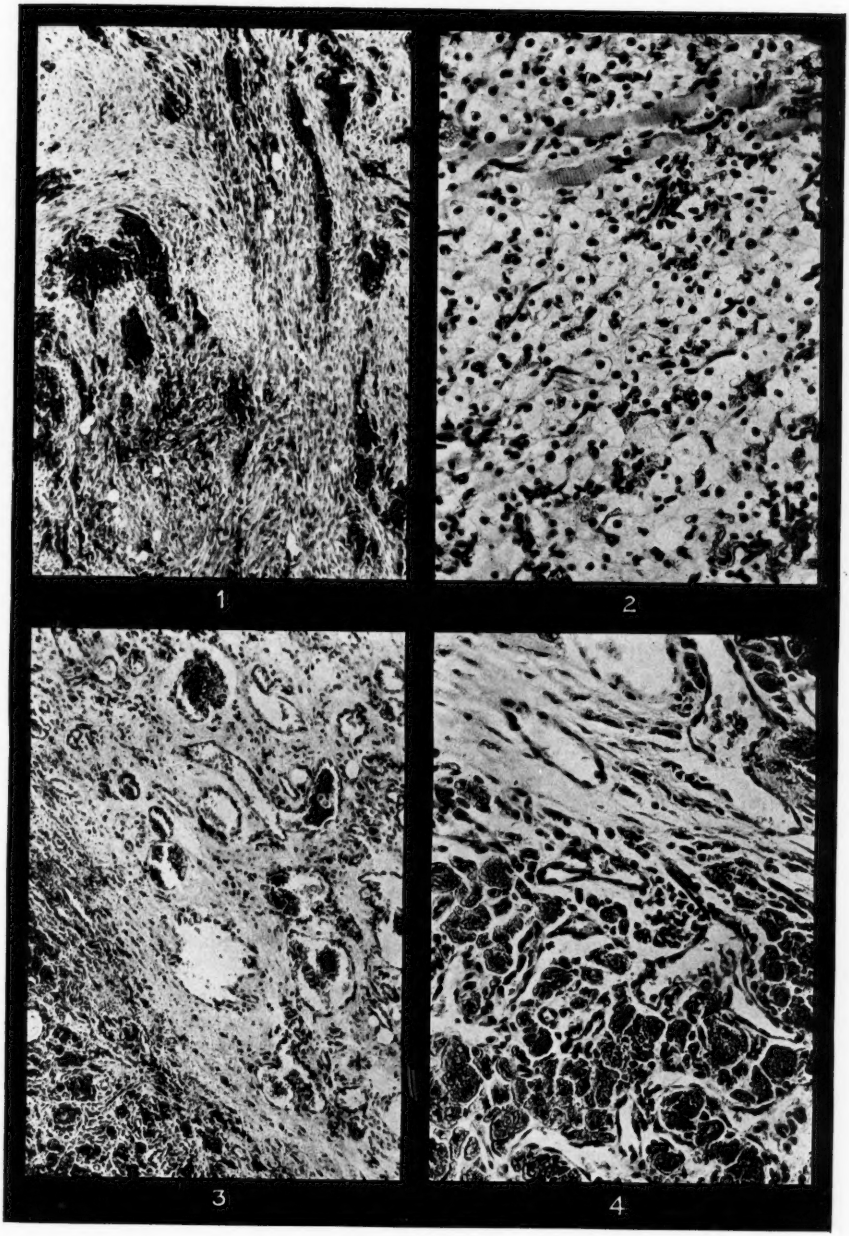
FIG. 1.—Development of sarcoma in association with thorotrast in a fibroma from tumour rat 21, killed 69 weeks after inoculation. The sarcoma consists of plump spindle-cells with hyperchromatic nuclei and no collagenous differentiation. The thorotrast is mostly contained within macrophages which have been caught up in the fibroma. × 75.

FIG. 2.—Histiocytoma from tumour mouse 7. The tumour consists of large clear cells with foamy cytoplasm and relatively small, dark nuclei. The tumour extends through the *panniculus carnosus*, shown at the upper part of the figure. × 275.

FIG. 3.—Angeio-endothelioma from tumour mouse 9. The tumour is composed of vascular spaces lined with plump endothelial cells and divided by wide trabeculæ of delicate fibrous tissue. The thorotrast deposit, shown at the lower part of the figure, is invaded by tumour capillaries as shown under a higher magnification in Fig. 4. × 75.

FIG. 4.—Angeio-endothelioma from tumour mouse 9. The thorotrast deposit is invaded and divided into lobules by the capillary angeio-endothelioma. × 250.



Selbie.

tumour that it can only be concluded that this is a development of sarcoma from the pre-existing fibroma by the continued action of the thorotrast.

There is evidence of the formation of a second tumour in association with thorotrast in 5 rats.

No. 21 has already been mentioned.

In tumour rat 14 the main tumour is at one end of the thorotrast deposit, which is divided into several nodules and surrounded by dense fibrous tissue. In one of these there is a sarcomatous area consisting of closely packed, long spindle-cells with hyperchromatic nuclei and numerous mitoses.

In tumour rat 18 a second spindle-cell sarcoma appeared 6 weeks after the first had been removed by operation. It developed in the thorotrast deposit which had been left *in situ* at the operation. Since autoplasts of the first tumour had grown to a considerable size (3 cm. in diameter) before it appeared it was unlikely that the second tumour was a recurrence. Moreover, the histological characters of the two tumours are distinct, the tumour-cells being smaller and more irregular in the second tumour.

In tumour rats 19 and 20 there are accumulations of foamy cells in association with the thorotrast deposits. In both cases they are on the far side of the deposit from the main tumour. They consist of closely packed, clear, polygonal cells with small nuclei and foamy cytoplasm. In rat 20 there is invasion of this tumour-like mass by the main tumour. One might have considered these as adventitious collections of foamy macrophages had not a similar tumour occurred in the mice (*cf.* Fig. 2).

Autoplasts.—Most of the earlier tumours grew with great rapidity and became ulcerated and infected at an early stage, so that the rats died of infection or succumbed to operative interference. Later it became easier to decide on the presence of a tumour, and by early operation the life of the animal could be considerably lengthened. In all but one case (No. 21) the autoplasts were successful when the rat lived more than 10 days after autoplasty was performed.

Transplantation.—Transplantation into normal rats was attempted with 13 tumours. Four were unsuccessful (Nos. 3, 7, 17 and 20). Only temporary tumours were obtained with 4 (Nos. 10, 11, 13 and 18). Five tumours were

TABLE II.—*Tumour Mice.*

Number.	Tumour noted (weeks).	Result of autoplasts.	Result of transplantation.
1	39	+	+
2	62	+	+
3	65	+	—
4	67	..	..
5	71	..	—
6	73	..	—
7	74	+	—
8	75	..	—
9	94	+	—

+ = positive.
— = negative.
Blank = not done.

successfully transplanted; No. 6 to the 5th generation, No. 12 to the 3rd generation, No. 14 to the 2nd generation, and Nos. 1 and 15 through one generation only.

Tumours in Mice.

Incidence.—The time of appearance of the tumours is shown in Table II.

The first tumour appeared at 39 weeks, and between 62 and 75 weeks 7 further tumours were observed. At 94 weeks a tumour was found in the last surviving mouse, making a total of 9 tumour mice. At 52 weeks there were 34 surviving mice and 1 mouse dead with a tumour, making a yield of 26 per cent. tumour mice in those surviving 1 year. At 62 weeks, however, when the second tumour appeared there were only 20 suriving mice, making a yield of 43 per cent. tumour mice in those living long enough to have tumours.

Description.—The mouse tumours, although considerably less in number, are much more varied in character.

Six of the mouse tumours are spindle-cell sarcomata, all of which are poorly differentiated. In all 6 tumours there is invasion of the *panniculus carnosus*.

In tumour 1 there are large amounts of thorotrast scattered throughout its substance, mostly in macrophages or lying loose in the intercellular spaces. However in some parts the thorotrast has been ingested by the tumour-cells—a feature which has not been observed in any of the other tumours.

The three remaining mouse tumours are an osteo-sarcoma, a histiocytoma and a capillary angeio-endothelioma.

The osteosarcoma (No. 5) appeared as a rapidly growing nodule just at the upper limit of the thorotrast deposit, and had apparently started in a rib. It consists in parts of lobules of osteoid tissue surrounded by narrow trabeculæ of sarcoma-cells, and in other parts of sheets of actively dividing tumour-cells. There is also invasion of the skeletal muscles.

The histiocytoma (No. 7) was about 1·2 cm. in its greatest diameter, and on section presented a smooth, opaque, white surface, permeated with thorotrast from one end where it was in contact with the thorotrast deposit. It consists of large clear cells with foamy cytoplasm and relatively small nuclei (Fig. 2). The tumour-cells have invaded the thorotrast deposit, and in the tumour there are large numbers of thorotrast-laden macrophages. There is invasion of the tumour-cells through the *panniculus carnosus*, but a decision of malignancy on this point cannot be made in view of the invasive characters of the cells from which the tumour is derived. An autoplast grew to a considerable size (1 cm. in diameter) in 9 weeks, and is in all respects similar to the original tumour except that it has acquired a vascular and delicately fibrous stroma. As has already been mentioned, similar though smaller tumours were found in 2 rats.

The capillary angeio-endothelioma (No. 9) first observed 21 months after inoculation was only 1 cm. in its longest diameter after 7 weeks. It was very soft, friable and vascular and was embedded in the thorotrast deposit. The main tumour is composed of vascular spaces lined with plump endothelial cells, and divided by wide trabeculæ of an open meshwork of fibroblasts with delicate collagen fibrils. Between the skin and the tumour a collection of

thorotrast-laden macrophages is divided into small lobules by the tumour in the form of capillaries. These capillaries also extend into the normal tissue (Figs. 3 and 4).

Metastases were not found in any of the tumour mice, but in Nos. 2 and 3 there was extensive intraperitoneal dissemination through the abdominal wall after operation, with invasion of the kidney in No. 3.

Autoplasts.—In all 5 cases where autoplasts were done they were successful. They all grew progressively except in No. 9, where they regressed after 3 weeks.

Transplantation.—Transplantation to normal mice was attempted with every mouse tumour except No. 4. No. 2 has now been transplanted to the 13th generation in mice. No. 1 was successful in the first transplantation but failed in the second. None of the remaining tumours grew on transplantation.

Early Stages of Tumour-Formation.

In order to study the changes in the tissues following an injection of thorotrast, 12 rats were inoculated subcutaneously with 0·05 c.c. on either side of the mid-ventral line. The rats were killed at intervals, and the resulting thorotrast deposits were removed for histological examination.

At 24 hours the thorotrast is mostly lying loose in the areolar tissue, and invaded by large numbers of polymorphonuclear leucocytes and by a few mononuclear cells. A number of polymorphonuclear leucocytes and a few mononuclear cells contain thorotrast granules.

At 48 hours very few polymorphonuclear leucocytes are seen, and most of the thorotrast is contained in macrophages. The blood capillaries are dilated and their endothelial lining swollen. There is already some development of fibrous tissue at the periphery. Many of the young fibroblasts contain thorotrast granules, which are found in clumps in the cytoplasm at either end of the nucleus.

At 6 days the thorotrast deposit consists of a mass of closely packed thorotrast-laden macrophages in which there are a number of capillaries and some fibrous tissue. It is surrounded by a capsule of fibrous tissue in which most of the fibroblasts contain thorotrast (Fig. 5). The appearances suggest that these fibroblasts have been formed from macrophages that have already ingested thorotrast granules.

Subsequently the fibrous tissue increases in amount, forming a dense capsule. As the thorotrast-laden macrophages die off the thorotrast is taken up by fresh macrophages, and so there is a certain local movement of the thorotrast. The centre of the thorotrast deposit, however, with the increasing fibrosis becomes necrotic and the final appearance, shown in Fig. 6, is produced. The centre is composed of an acellular granular mass of thorotrast surrounded by thorotrast-laden macrophages. Bands of hyaline connective tissue are found traversing the nodule, and the nodule is surrounded by dense connective tissue. Thorotrast is also found in the surrounding connective tissue, mostly ingested by macrophages, of which all stages can be seen from the relatively small mononuclear cell to the fully packed macrophage. Thorotrast-laden macrophages can usually be found in the lymphatic glands, more often and in greater quantity in the axilla than in the groin. The amount found in the

glands, however, is usually very small, indicating that very little thorotrast is removed from the site of inoculation. In only one case was thorotrast found elsewhere, namely in the liver of tumour rat No. 10.

The earliest stage of tumour-formation to be detected in this series is in a rat killed 72 weeks after inoculation. Partly surrounding a thorotrast nodule there is an area of actively proliferating cells with hyperchromatic nuclei (Fig. 7). From its situation it can be seen that it has arisen from the fibrous tissue capsule of the deposit, which consists of loose thorotrast lying in a network of hyaline tissue.

In another rat also killed at 72 weeks there is a small fibroma extending through the subcutaneous areolar tissue to the corium (Fig. 8). It consists of loosely packed interlacing bands of well-differentiated fibroblasts, including a number of thorotrast-laden macrophages. It has no close relation to any large quantity of thorotrast, and thus shows that a small amount may cause a tumour.

In none of the other rats killed at the end of the experiment has evidence of early tumour-formation been found, but in several of these there is considerable fibroblastic proliferation. Fibroblastic proliferation is noticeable in many of the tumour rats, suggesting that tumours are formed in rats which give a vigorous inflammatory reaction to the presence of the thorotrast. This is well shown in tumour rat 14, which has already been mentioned as presenting a second early tumour.

DISCUSSION.

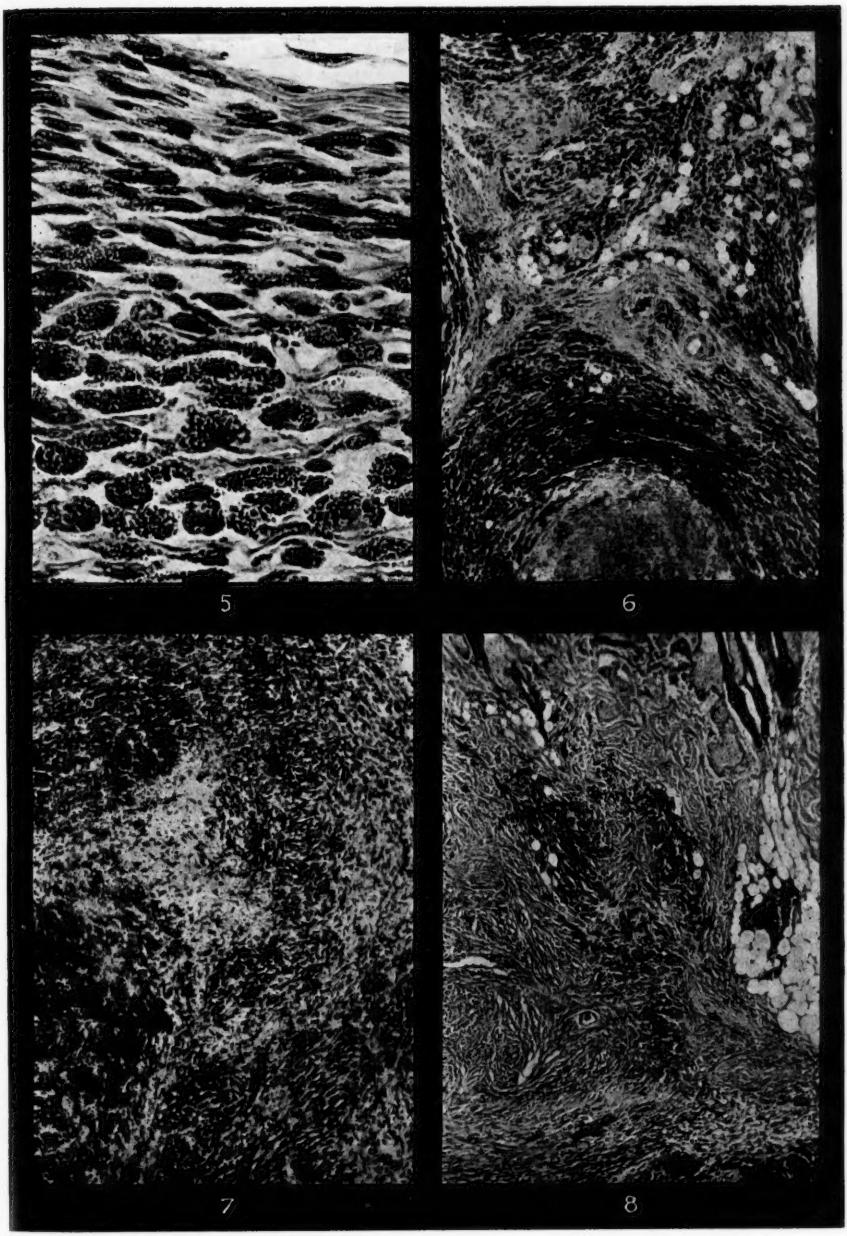
Roussy, Oberling and Guérin (1936*a*, 1936*b*) have recently described further experiments with thorotrast in rats, mice and fowls. They find a correlation between the amount of thorotrast injected and the number of tumours produced in rats surviving 1 year. In 3 series of rats injected with 5·0 c.c., 2·5 c.c. and 0·5 c.c., tumours were produced respectively in 100 per cent., 71 per cent. and 42 per cent. They failed to obtain tumours in mice or fowls. It is evident, however, that the percentage of tumours formed does not fall proportionately with the dose, and that much smaller doses would produce tumours.

FIG. 5.—Thorotrast deposit 6 days after subcutaneous inoculation in the rat. The nodule consists of thorotrast-laden macrophages surrounded by newly-formed fibrous tissue. Most of the fibroblasts contain thorotrast granules, which are found in clumps in the cytoplasm at either end of the nucleus. × 500.

FIG. 6.—Thorotrast nodule from tumour rat 21, killed 69 weeks after inoculation. The centre of the nodule is composed of an acellular granular mass of thorotrast in a network of hyaline tissue. This is surrounded by a capsule of dense connective-tissue. There is a considerable fibrosis of the surrounding tissue in association with thorotrast-laden macrophages. × 55.

FIG. 7.—Early sarcoma in a rat killed 72 weeks after inoculation. The sarcoma, which can be distinguished as composed of cells with darkly-stained nuclei, is situated in the fibrous tissue forming the capsule of a thorotrast deposit. The latter, shown at the left of the figure, consists of loose thorotrast lying in hyaline tissue. × 85.

FIG. 8.—Early fibroma in a rat killed 72 weeks after inoculation. The tumour is situated between the *panniculus carnosus* and the corium, and includes a number of thorotrast-laden macrophages. × 55.



Selbie.

It has also been found that tumours can be produced in a fairly large proportion of mice (43 per cent.) surviving 14½ months after injection. The latent period in mice is much longer than would be expected, and is probably due to the relative insensitivity of the connective tissue of the mouse compared to the rat. In the experiments of the French workers the mice all died within 1 year, whereas in the present series only 1 tumour had appeared in that time.

Considering the low radio-activity of thorotrast its ability to produce tumours is surprising. It seems to act primarily as a foreign body, causing a continuous reaction and proliferation of the connective tissues, which, in turn, by the constant bombardment of alpha particles, go on to tumour-formation. As has been shown, any of the types of cells involved in the granulomatous reaction, fibroblasts, histiocytes or capillary endothelium may form tumours. The process is probably analogous to the production of sarcomata in rabbits by the X-radiation of chronic inflammatory lesions induced by *Streptococcus caviæ* (Lacassagne and Vinzent, 1929) or diatomaceous earth (Lacassagne, 1933). This was successfully repeated in rabbits by Burrows, Mayneord and Roberts (1937), who used powdered silica to produce the inflammatory action. The amount of X-radiation used, 600 r, was insufficient to produce tumours in normal animals. The radiation was given in one dose, whereas, with thorotrast, radiation, though very much smaller in amount and differing in quality, is continuous. In assessing the probability of malignancy occurring after injections of thorotrast one must consider that it is not only a radio-active substance, but that it is also acting on an inflammatory tissue, produced by the thorotrast and probably much more susceptible to radiation than a normal tissue.

SUMMARY.

Tumours can be readily induced in rats and mice by the subcutaneous injection of thorotrast.

The proportion of tumours induced in rats surviving 52 weeks after the injection of 0·6 c.c. thorotrast has been 58 per cent., and the proportion of tumours in mice surviving 52 weeks after the injection of 0·2 c.c. thorotrast has been 26 per cent.

It is suggested that the carcinogenicity of thorotrast is due not only to its radio-activity, but also to the susceptibility of the inflammatory tissue which it produces.

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OBSERVATIONS ON LIVING VACCINIA AND ECTROMELIA VIRUSES BY HIGH POWER MICROSCOPY.

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OUR conceptions of the morphological characters of a number of the larger viruses have been clarified by the work of Barnard (1931), who, by employing optical systems of higher resolving power than any previously used in the field of virus research, and by the use of fresh, unstained, unfixed material, has shown conclusively, for example, that the inclusion bodies found in infectious ectromelia represent virus colonies composed of uniform units, the elementary virus bodies. By the application of direct and dark-ground methods of illumination to ultra-violet microscopy Barnard has succeeded in showing these and a number of other virus bodies as true images and not merely as unresolved points of light. The images obtained show that the virus bodies so far examined by him are very similar to ordinary micro-organisms in their form and in their natural arrangement in infected tissues.

Barnard has shown too that valuable work can be done in visual microscopy by the ordinary dark-ground method. He has demonstrated that by this method the elementary virus bodies of infectious ectromelia can be clearly seen as elements of a constant order of size, and having a characteristic distribution within the inclusion body. In this work on infectious ectromelia he used sections of frozen tissues, inclusion bodies which had been freed from tissues, and cell-free filtrates of infected tissues.

Bland and Canti (1935) also used the visual dark-ground method with considerable success in their studies on psittacosis. They were particularly interested in the relationship of virus to cell and, using infected tissue-cultures, were able to demonstrate a number of physical characteristics of the growing psittacosis virus.

In the early stages of the present investigation attempts were made to study the vaccinia virus as it occurs in infected tissue-cultures but with little success. The ordinary visual dark-ground method of examination was used. Then attention was turned to the lesions produced by vaccinia and ectromelia viruses in the chorio-allantoic membranes of developing duck and fowl

embryos. At first fragments of membrane containing virus lesions were mounted in saline as thin, coverslip preparations. Results were encouraging, but in view of the rapidity with which such preparations deteriorated, and the difficulties encountered when attempts were made to trace successive stages in developing lesions, it was determined to explore the possibility of using annular oblique incident illumination in the examination of the living, undisturbed, infected chorio-allantoic membrane whilst it is still *in situ* in the egg. Illumination was by means of the Heine Ultropak (Leitz) which had proved useful in the preparation and examination of tissue-cultures under low powers (Himmelweit, 1933). Similar apparatus had also been used by Kaiser and Vonwiller (1933, 1934) in their study on supra-vitally stained vaccinia lesions of the rabbit cornea. These workers appear, however, not to have been able to differentiate with certainty the structures to be seen in infected cells.

First attempts to use the Ultropak gave images of low contrast and intensity even at magnifications as low as 500 diameters. However, by the introduction of a subsidiary illuminating system,* images were improved in quality, and structures were revealed which previously had been hidden by the glare caused by the opalescence of the material under examination.

Annular oblique incident illumination affords certain advantages in studies of highly opalescent biological material, of which cells and tissues are outstanding examples. Images of structures lying on or immediately below the surface suffer comparatively little interference from underlying structures. This is partly because these deeper structures are comparatively poorly illuminated. In dark-ground and transmitted light systems conditions are reversed. In them the light is thrown up from below and it is the deeper structures which are first illuminated and with fullest intensity.

The image itself, which is obtained by annular oblique incident illumination, though essentially similar to that obtained by ordinary dark-ground illumination, differs in that there is a superimposed, though less intense image similar in character to that obtained by direct vertical illumination, the total effect being to produce a plastic image.

A major difficulty arose from the fact that the technique of egg inoculation necessitates the production of an artificial air-sac (Burnet & Galloway, 1934) so that the infected portion of the chorio-allantoic membrane, bearing the lesions to be examined, forms the base of the sac and lies at a considerable distance from the egg-shell. Moreover, the infected portion of membrane may exhibit surface irregularities. Thus, before objectives of high power, and consequent short working distance, could be used it was necessary to find how to flatten out the membrane into an even plane and reduce its distance from the microscope objective without damaging or displacing cells. A satisfactory method was found and is described below.

* This system is one developed by Mr. J. Smiles at the National Institute for Medical Research in connection with his own studies of calcified opalescent material. He proposes to publish a detailed description of the system in the near future and to discuss, at the same time, the optical principles involved in the application of annular oblique incident illumination to the study of biological material. It is with great pleasure that I express my thanks to Mr. Smiles for expert advice and help on the optical side of the work reported in this paper.

TECHNIQUE.

(1) *Egg Inoculation.*

The method of inoculating the egg with infectious or other material is essentially the same as that described by Burnet and Galloway (1934), and Burnet (1936).

A modification, however, has been introduced with regard to the operation of separating the chorio-allantois from the shell-membrane. Instead of applying suction on the opening into the natural air-sac of the egg after the piercing of the chorio-allantoic membrane, it has been found, following the suggestion of Sir Patrick Laidlaw, that the application of continuous suction by means of a rubber teat (started before the beginning of the operation) facilitates the separation of the membranes without the occurrence of hæmorrhage.

(2) *Preparation of the Egg for Microscopical Observation.*

After the inoculated egg has been incubated for a predetermined period the shell and shell-membrane forming the roof of the artificial air-sac are removed, leaving a margin of only 1 to 2 mm. above the attachment of the chorio-allantoic membrane, but taking care not to injure the membrane. Next, on the surface of the shell, in the form of a ring round the margin of the hole and slightly overlapping the broken edges of the shell, a little wall of wax is built up. A convenient wax consists of paraffin wax (M.P. 56° C.) and vaseline in equal parts. It is used just hot enough to flow and time is given for one layer to solidify before the next is laid upon it. Usually the wax wall has been built up to a height of some 2 mm. above the cut edge of the egg-shell and has had a breadth or thickness of about 5 mm. Then, after a few drops of saline have been placed on the exposed surface of the chorio-allantoic membrane, a thin glass coverslip, about $1\frac{1}{2} \times 2$ inches in size, is sealed on top of the wax wall making the chamber perfectly airtight. To make the membrane completely accessible for examination and at the same time to level out its surface irregularities, the membrane must be raised till it comes into close contact with the underside of the coverslip-roof of the chamber. The method is to reverse the process by which the artificial sac was formed. By refilling the natural sac with air one can force all air from the artificial sac, obliterating its cavity. It will be remembered that when the egg was being prepared for inoculation a hole was made in the wall of the natural air-sac. This hole still exists at the end of the egg though the sac is collapsed. Now a foot or so of rubber tubing is fitted to a short glass tube and the free end of this glass tube is securely sealed over the hole in the sac by means of "Ash's Model Cement ".* This arrangement provides an easy means of blowing air into the natural sac. But before air can escape from the artificial sac and allow the membrane to move upwards, a hole must be made through the wax wall which seals coverslip to shell. A hot wire is used for the purpose and the hole is made as close as possible to the under-surface of the coverslip. With the hole made, all is

* Supplied by " The Amalgamated Dental Co., Ltd.", London, W. 1.

ready for the gentle inflation of the natural air-sac. The chorio-allantoic membrane approaches the under-surface of the coverslip, one tilts the egg away from the opening in the wall and presently all air has been expelled and the saline, which, as already described, was placed in the sac at an earlier stage in these operations, begins to flow through the opening. Pressure is maintained until the membrane is in close contact with the coverslip and most of the saline has been forced from the chamber. Then the opening in the wax wall is sealed by means of the hot wire so that on releasing the pressure exerted by the air in the now fully inflated natural sac, the chorio-allantoic membrane remains in close contact with the coverslip through which all microscopical observations are made.

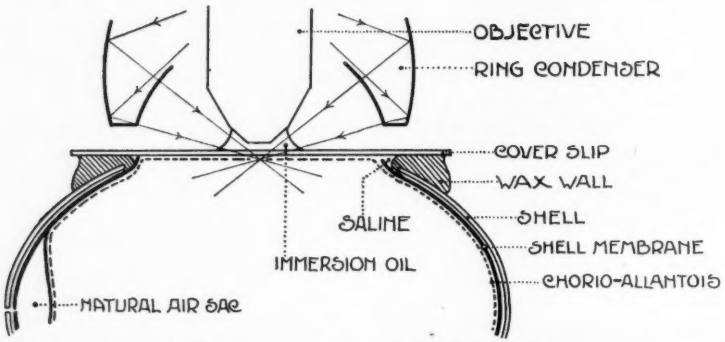
Many observations have been made on fowl embryos, but as a rule duck embryos have been preferred on account of their somewhat larger size. The embryos have usually been inoculated between the 9th and 15th days of incubation. Young embryos offer an advantage in that in them observations can be carried over longer periods, for whatever the age at inoculation, after the growing embryo has reached a certain age and size the chorio-allantoic membrane cannot be usefully examined by the methods described. In young embryos, also, the chorio-allantoic membrane is extremely thin and delicate in structure, whereas as growth proceeds, the membrane becomes thicker, more cellular in structure, and more gross, with an increasing opalescence and consequent loss of delicate detail in the microscopical image.

(3) *Virus Suspensions.*

Two viruses have been used. These are (*a*) a dermal strain of vaccinia, isolated from calf-lymph, and (*b*) the original Hampstead strain of infectious ectromelia (Marchal, 1930) isolated from a mouse liver. Each of these viruses was passed about 20 times on duck-egg membranes before being used in the present investigations, and during the investigations they were carried through a further 30 or more similar passages. During all this time both remained absolutely stable. In general it was found desirable that virus suspensions should be of such a strength that when the usual 0·1 c.c. was used for the inoculation of a chorio-allantoic membrane, 5 to 20 well-defined nodules would be produced. Suspensions of this desired strength were commonly obtained with either virus when 3 to 6 nodules, 3 to 5 days old, were emulsified in 3 c.c. of a saline-broth mixture (equal parts of each) which was then diluted to 1 in 10,000 with saline. Dilutions of 1 in 1000 usually produced confluent lesions which only rarely proved useful for microscopical examination. Incubation of the inoculated egg has been at 37° C., and has varied from 1 to 7 days in duration. In young lesions infection is confined to the uppermost cell-layers of the membrane and observations can be made without previous manipulations. In more advanced lesions most of the more recently infected cells lie deep and are overlain by the necrotic cells and degenerated leucocytes belonging to older parts of the lesion. Often, these necrotic masses can be removed with fine forceps, getting rid of unwanted opalescent material and at the same time allowing the advancing edge of the lesion to be pressed more closely against the coverslip.

(4) *Optical Equipment.*

The optical equipment finally adopted consisted of a Heine Ultropak outfit supplied by Leitz. The objectives ranged from × 22 to × 100, the latter being an oil immersion of 1·0 N.A. The Ultropak reflecting condenser has been used throughout, but the light-collecting systems supplied with the Ultropak were replaced by a long-focus condenser (see footnote on p. 109) in combination with an arc lamp, a Pointolite, or an Osira high-pressure mercury vapour lamp (General Electric Company) from which the covering opal bulb had been removed, leaving the lamp itself exposed. Reference to



TEXT-FIG. 1.—Diagram of the conditions which obtain when an egg-membrane is flattened against the under-surface of a coverslip for examination by annular oblique incident illumination.

Text-fig. 1 will perhaps make clear some of the conditions which obtain when a membrane preparation is under microscopical examination.

Occasionally, when for special purposes ordinary dark-ground illumination was needed, the 1·2 N.A. dark-ground condenser of Leitz, in combination with the 1/12*a* oil immersion by the same makers, was used.

THE NORMAL CHORIO-ALLANTOIC MEMBRANE OF THE DUCK EGG.

Under low magnification the membrane appears as a uniformly yellow field in which the vessels, large and small, can be traced, but in which little else can be made out. The movement of blood-cells can be recognized with ease, and arteries can be distinguished from veins by the character of the blood-flow—intermittant in arteries, continuous in veins. Under high powers the uppermost layer of the ectoderm can be found readily. In it the epithelial cells appear as regular polygonal structures with slightly rounded corners. The cell-margins are not sharply defined, but are indicated by fine lines of small, highly refractile globules. Under normal conditions the cytoplasm of

the epithelial cells is almost structureless, appearing as optically homogeneous material somewhat opalescent in nature. Usually a dark structureless, round or oval area representing the nucleus can be detected in the middle of the cell.

At times, when, in young embryos, the uppermost layer of membrane cells is sharply in focus, no vascular structures can be clearly seen. Only when search is made in deeper layers do blood-vessels come sharply into view. Then a useful image of the interior of the vessels is obtained, and this in spite of the fact that the vessels are being seen through a number of cell-layers. The sharpness of the image is due to the optically homogeneous nature of the epithelial cells. Vessels in older membranes give a clearer, more contrasting image, since, as the result of developmental changes, they have come to lie at a more superficial level (Danchakoff, 1917). Diapedesis is a common feature; possibly due to the intense light of the microscope illuminating system. After wandering from the blood-vessels, the leucocytes may be recognized in the neighbourhood of extra-vascular structures.

The chorio-allantoic membrane of the domestic fowl differs slightly from that of the duck. All cells are smaller and the nuclei of epithelial cells, instead of appearing as dark, empty spaces, show up as round or oval, slightly opalescent structures.

LESIONS OF INFECTIOUS ECTROMELIA IN THE CHORIO-ALLANTOIC MEMBRANE OF THE DUCK EGG.

Under low magnifications the edge of a well-formed ectromelia nodule is clean cut. In this it differs from a vaccinia lesion, the difference being associated with the fact that in ectromelia there is a less extensive infiltration with leucocytes. In both diseases the lesions are generally situated on, or very near, the larger blood-vessels. The lesions may grow along the vessel and may even extend along the subsidiary vessels. This suggests that in both ectromelia and vaccinia the virus attack falls primarily on some type of cell belonging to the vessel wall. It has not been possible, however, to observe the very early stages of infection in either disease, but in view of Ledingham's studies (1927) on vaccinia it seems very probable that in ectromelia, as in vaccinia, the main incidence is on cells of the reticulo-endothelial system. Soon both epithelial cells and cells of the mesodermal layer are also attacked, a fact which can be demonstrated with ease in membrane preparations.

In ectromelia the first visible evidence of an infection is the presence of infiltrating leucocytes which move about in intercellular spaces or lie on the surface of epithelial cells. An increased number of leucocytes is noticeable within the capilliaries of the disturbed areas. Another early sign of infection is a general increase in opacity in the infected areas. This increase has several causes. Leucocytes are more opaque than tissue-cells and their presence in abnormal numbers accounts for some of the increase. The epithelial cells of the area become more opaque. There is an increase in the number of fat-droplets which they contain and the cytoplasm appears to change, for the cells now look slightly enlarged and denser.

In the period immediately following inoculation virus bodies have not

been observed, but at the end of the first 24 hours a number of cells contain one or more tiny inclusion bodies, the smallest being only about 1 to 2μ in diameter. An outstanding feature of ectromelia infection in this, as in other stages of the disease, is the number of inclusion bodies commonly found in a cell. Quite frequently 5 or 6 or more are present, generally of various sizes. Cells containing inclusion bodies which are larger than the average always contain small ones in addition. Using the highest power objective available, 1·0 N.A., one can observe with certainty that even these earliest inclusion bodies are all composed of numerous unresolved granules which represent the elementary bodies. The particles are all of equal luminosity and this, since the particles are not resolved, is accepted as an indication that the elementary bodies are all of one order of size. In older inclusion bodies, which may have a diameter of as much as 8μ or so, the granules are tightly packed, but in the very small, younger bodies described above, the granules are packed less tightly and are often seen arranged at regular intervals along parallel arcs or closed curves, a formation which Barnard has described as " line arrangement ".

A phenomenon of old lesions is the confluence of one or more inclusion bodies to form giant structures. These giant forms are generally irregular in outline and are often found lying free amid the remains of disintegrated cells. They may reach a diameter of 30 to 50μ. The actual process of fusion can be directly observed by mounting scrapings from the uppermost layers of a well-developed ectromelia lesion in saline on an ordinary microscope slide and examining by the ordinary dark-ground method. In a successful preparation a fair number of inclusion bodies are seen within cells which are still apparently living. In addition there are a number of extra-cellular inclusion bodies which may be either stationary or floating freely in the mounting fluid. There are also present a large number of very small granular elements which in luminosity are all similar to one another and to the particles found in the inclusion bodies, and for this reason, amongst others, it is evident that these scattered particles are actual elementary bodies. When in these preparations extra-cellular inclusion bodies come into close contact with one another they tend to adhere and form larger units to which free elementary bodies readily attach themselves. Giant formations may develop in fields where only free elementary bodies have been present with no original inclusion body to act as a nucleus of aggregation. By successive accretions giant formations may grow to such a size that they may more than cover the oil immersion field (Leitz 1/12*a* objective with × 10 ocular).

This phenomenon of fusion permits certain inferences to be drawn concerning the nature of the matrix in which the elementary bodies were originally embedded in the intra-cellular inclusion bodies. It must be that in these preparations of lesion-scrapings each free elementary body is still surrounded by a coating of the original matrix which cannot be readily soluble in saline or it would have disappeared, and which must be in some sense " sticky " in nature or aggregation would not take place. It cannot be simply that the virus particles themselves attract one another for even in the giant formations they seem never to come into actual contact with one another ; in fact the amount of separation is relatively considerable, being within the limit of resolution of

the observing system. That the matrix cannot be very fluid is suggested by the fact that Brownian movement of elementary bodies is never seen in the interior of inclusion bodies. Further, there is evidence that the matrix of the intracellular inclusion is not of high rigidity. Occasionally a relatively large inclusion may be seen in a long, extended process of a connective tissue cell (Fig. 4). In such a case it may be noticed that the inclusion has become deformed to fit the shape of the process, not *vice versa*. Another observation points to a certain fragility of the matrix. In smears it is not very uncommon to find extra-cellular inclusions which have become crushed during the making of the preparation, liberating a shower of inclusion bodies. Barnard, using mouse tissues, has remarked on the firmness of the matrix in ectromelia inclusion bodies. Possibly there are differences due to the species of host infected.

VACCINIA LESIONS IN THE CHORIO-ALLANTOIC MEMBRANE OF THE DUCK EGG.

The vaccinia lesion consists of a mass of infected cells and infiltrating leucocytes, heaped up above the level of the still unchanged membrane. Many of the cellular elements are in process of disintegration and for this reason the mass is highly opalescent and gives images of low contrast and lacking in detail. Yet even here it is possible to distinguish internal structures in some of the most superficial cells of the mass. Towards the periphery of the nodule the vertical depth of the cell-mass decreases and there is a corresponding fall in opalescence. The edge of the mass rises rather sharply from the membrane level and has not the smooth contour of the ectromelia lesion, but can best be described as ragged, loose, crumbly peripheral tissue from which many tiny masses of cells project and overhang. The cells in these excrescent masses are clearly visible. They are immediately surrounded by saline which itself has low scattering power, and because of their relative isolation the cells give images which are little disturbed by light scattered from neighbouring structures. Moerover, they have not, as yet, undergone disintegration, and they form ideal material for the study of cellular phenomena related to the infective process.

Although the work of Ledingham (1927) suggested that in vaccinal infection of the rabbit the virus attacks, primarily, cells of the reticulo-endothelial system, it is the epithelial cells of the infected chorio-allantoic membrane which show the most characteristic evidence of infection. This may be partly because of their accessibility for observation. The epithelial cells are rounded in shape and vary in size, some being very much larger than normal. This increase in size was noted in stained sections by Unna (1894), who described the phenomenon as " ballooning colliquation ". Cells in this condition are very conspicuous when the membrane lesions are examined by annular oblique incident illumination. The plasticity of the images obtained by this method is particularly well adapted to an appreciation of the globular character of these cells and the distribution of cell-contents. Ballooning may take the form of a general increase in size of the cell, more or less uniform in all directions, or it may take the form of a swelling of only a localized part of the cell.

The epithelial cells of the vaccinial lesion, so far as they can be examined *in situ*, which as already mentioned is only at the very surface of the mass, appear not to be directly in contact with each other. There seems to be a narrow but definite gap between, and this, presumably is filled by an " intercellular " substance.

No attempt will be made to describe all the multitudinous appearances which are to be found during the progress of an infection, but attention is directed to some of those features which occur with regularity and which appear to indicate the mode (*a*) by which infection spreads within the cell, and (*b*) by which at least some of the virus is liberated from the cell.

Virus bodies are first seen on the second day following inoculation. At this time a narrow peripheral zone of vibrating particles can be found in a number of the cells of an infected area. On account of their high and uniform brilliance, and in view of the phenomena to be observed during the progress of the disease, there is little doubt that these particles are Paschen bodies. In the cell, they exhibit constant Brownian movement and are apparently suspended in a matrix of low viscosity which is not separated off from other cell-contents by any true membrane or similar sharp division. The occupied zone is sometimes fairly complete and then envelops the cell much as a very thin cortex might do. More often the zone is incompletely invaded and the distribution of virus within the zone is patchy. In an extreme instance of patchy distribution, quite often seen, the appearance which this peripheral zone presents in optical section is that of a row of droplets of various sizes containing a small number of virus bodies.

This indefiniteness of form makes description difficult. " Inclusion body " usually connotes a more or less concrete and well-defined intra-cellular formation, but here in vaccinia the appearance is more like that of a shoal of fish in an ever-extending pond. For want of a better term, " accumulation of virus " is used to denote the collection of elementary bodies together with the enveloping matrix.

As the infection progresses the accumulations of virus increase in size. They penetrate more deeply into the cytoplasm and, extending laterally, may join up with neighbouring accumulations. Increase in virus numbers is always accompanied by a corresponding increase in the volume of the matrix so that the concentration of virus in the matrix remains fairly constant in all stages of a cell infection. The inner boundaries of the expanding virus accumulations, which still tend to range themselves in cortical sheets of varying thickness and extent, are generally irregular owing to distinct inequalities in the rate of virus penetration of cell-cytoplasm at different points. In some cases narrow processes or bridges of vibrating virus bodies unite the originally isolated accumulations. It is not uncommon to find processes entering the cytoplasm and passing deep into the cell.

Increase in the volume of virus accumulations continues until the greater part of, or even the whole of the cell cytoplasm appears to be filled with vibrating virus bodies. The nucleus alone escapes as it does in ectromelia. An optical section through a diametral plane of the cell may now show the virus accumulation as a half-moon or triangular-shaped area bounded by the surface of the cell and by the nucleus. Focusing above and below this plane shows that

the virus accumulations envelope the nucleus. These appearances, the result of complete or nearly complete invasion, are found with greatest frequency at the height of the disease. They are then to be found in a large number of cells. Even at this time, the 3rd or 4th day following inoculation of the membrane, there are cells near the edge of the lesion which are in the earliest stages of cell infection, a fact which suggests that within the lesion infection spreads from cell to cell radially from the centre towards the periphery.

Characteristically, intra-cellular particles in vaccinia are in constant vibratory motion, but occasionally, in any stage of the disease, but particularly at its height, immobilization of the particles may be observed. The process from start to finish may extend over many hours. Cells observed in an intermediate stage contain areas in which vibration has ceased and areas in which vibration is still active. Such cells are frequently met with. The cessation of Brownian movement can only be due to increase in the viscosity of the matrix, and this increase presumably is an accompaniment of the death of the cell. Cessation is apparently independent of temperatures ranging from 10° to 40° C., as for example both at room temperature and at incubator temperature microscopic fields have remained under observation for days without change in particle vibration being detected.

A new phenomenon which indicates a mechanism by which some of the virus is liberated from the cell has been observed. In intercellular spaces of the lesions and in the fluid at the edge of the lesions certain globular bodies, containing vibrating granules, are seen in the later stages of an infection. The bodies vary in size, the largest having a diameter of about 15μ. Occasionally one of these bodies is found connected with an infected cell by a strand which also contains vibrating particles. The concentration and luminosity of the particles vary slightly from one body to another, though in any one body the luminosity is constant. In some the luminosity of the contained particles is greater than that of the vibrating intra-cellular virus bodies normally found in infected cells, but generally the luminosity is either equal to or less than that of the particles which are within cells.

The available evidence indicates that these globular formations are really extensions from infected cells ; a herniation containing virus bodies suspended in a matrix of low viscosity occurs in a localized area of a cell in consequence of " ballooning colliquation " and presently becomes separated from the rest of the cell. The evidence for this interpretation of the findings is as follows : The vibrating particles which are present in the " extrusion bodies " are similar in " population density " and in luminosity to the vibrating virus particles to be seen in the localized swellings and bulging protrusions of cells undergoing " ballooning colliquation ", and similar to the particles in the strands which, as mentioned above, may sometimes be observed connecting infected cells to " extrusion bodies ". During the development of hernial swellings in " ballooning colliquation " the luminosity of the contained virus particles is generally reduced. This change is more suggestive of change in the optical properties of the matrix than of change in the physical character of the virus particles, but the main point is that by this change in luminosity recognized virus particles take on exactly the same visual characters as those exhibited by the particles found in " extrusion bodies ". Thus one may safely

say that in spite of differences in the luminosity of the particles within the different extrusions they are identical with the virus bodies present in infected cells.

The fact that virus bodies within an extrusion body do not immediately disperse is taken as an indication that the matrix of these bodies is not readily miscible with saline.

Concerning the nature of the Guarnieri body, it may be mentioned that no structure closely resembling such bodies, other than the virus accumulations already described, has been observed in the cells. Within the first 48 hours following inoculation of the membrane no structures are found other than those seen in normal cells, with the exception of the elementary virus bodies. A large dark area representing the nucleus occupies a central position in the cell, and in the cytoplasm there are a few globules of a fatty or lipoidal nature. After the second day there is an increase in size and number of fat-globules, the extent of the change differing from cell to cell. Changes in the nucleus are found from the third day onwards. The nucleus becomes highly opalescent and has, as a rule, an irregular, highly refractile, patchy surface. Occasionally this globular structure is lobulated and contains two smaller bodies which are rounded in shape and have smooth outlines. These smaller bodies are taken to be nucleoli. The structures described as belonging to the cell are at all times distinct from the elementary virus particles.

Only negative information can be gained concerning the size of vaccinia elementary bodies. Since they are unresolved by the optical system used (1·0 N.A. objective, wave-length of 439$\mu\mu$) they, like the elementary bodies of infectious ectromelia, must be below 0·22μ in greatest diameter.

DISCUSSION.

The use of tissue-cultures for the optical study of virus infections has certain drawbacks. It is not easy to grow suitably thin sheets of cells and prepare them for examination. Feeding operations are too apt to damage them and pH and other changes make prolonged observation under the microscope difficult. These disadvantages are not encountered when, as in the present investigations, the chorio-allantoic membrane is used. The metabolism is here maintained by the normal vascular system of the embryo and the various types of cells grow and differentiate and react under all the regulating mechanisms of an intact living organism. In fact the intact chorio-allantoic membrane represents an ideal "tissue culture", and changes within the infected membrane may be taken as very closely similar to changes which occur in a natural infection.

From the description which has been given of the normal chorio-allantoic membrane as seen under annular oblique incident illumination, it will be appreciated how readily the capillary bed and larger vessels are available under undisturbed natural conditions. One and the same microscope field can be kept under continuous observation for days together, an advantage hardly to be obtained in any other way. Changes can be observed in the rate of flow of the blood under various influences, and even changes in the

distribution and form of the cellular elements of the blood can be recognized. In an established vaccinia infection, for example, one glance at the capillaries in a normal portion of the membrane has often shown a large increase in the number of leucocytes in the circulating blood—a general leucocytosis in fact.

The ectromelia observations demand further comment. It will be recalled that in the older inclusion bodies the elementary virus particles are more tightly packed than in the younger ones. This difference suggests that in the growth of an inclusion body a stage is reached in which resistance to expansion becomes appreciable and, as a consequence, the multiplying virus bodies become more closely arranged. The common occurrence of multiple inclusions of similar size in a single cell suggests that commonly cells are simultaneously invaded by a number of separate units. That the invading units may sometimes vary in infectivity, by aggregation or otherwise, is suggested by the occurrence of inclusions of different size in a single cell; though an alternative explanation is that the smaller inclusions may result from a late spread of infective material from larger inclusions within the same cell.

In vaccinia the study of virus bodies in living undisturbed membrane lesions has extended our knowledge of the relationship existing between virus and cell in this disease. The evidence that the virus bodies have been correctly identified may be summarized as follows: First, similar bodies exhibiting similar characteristics are never seen in normal membranes, in membranes exhibiting non-specific lesions, or in membranes infected with ectromelia, herpes, or influenza viruses. Secondly, these bodies are of uniform size as judged by their uniform luminosity. Thirdly, the elementary particles of vaccinia and ectromelia both react in the same peculiar way in the presence of the fluorescent dyes, primuline and titan yellow; they take up the stains with like intensity and the stained particles, when exposed to ultra-violet light, fluoresce with the same intensity and colour, reactions which distinguish them from all other elements found in the tissues (Himmelweit, 1937).

The presence of vibrating intra-cellular particles in vaccinia was first described by Volpino in 1907, the year in which Paschen demonstrated the elementary bodies by a staining method. Volpino observed the vibrating particles in epithelial cells of the infected rabbit-cornea. Goodpasture, Woodruff and Buddingh (1932), Rhodes and van Rooyen (1937), and Tang and Wei (1937) have observed similar dancing particles in fibroblasts and epithelial cells of tissue fragments obtained from vaccinal lesions of rabbit cornea or of the chorio-allantoic membrane from the developing egg.

What is the relationship of these vibrating particles to the Guarnieri body? The typical Guarnieri body, as revealed by staining methods, cannot always be found in tissues in which virus can be demonstrated in the form of Paschen bodies or only by animal tests for infectivity. This failure to find the Guarnieri body wherever virus is present probably arises from the fact that only comparatively large globular accumulations of virus are characteristically identifiable by staining methods. Observations show that such characteristic accumulations are not always present in a membrane lesion at

every stage of the disease. In the very earliest stages the virus is present in such small amounts and is distributed in such a way that staining methods must fail to demonstrate these as being clearly of the same essential nature as the Guarnieri bodies. Only in intermediate stages of the disease, and under some unknown favourable condition of the cytoplasm of the cells, do the accumulations of virus become sufficiently massive and typical in shape to be identifiable as undoubted Guarnieri bodies. Probably fixing and staining processes play a part in producing the typical Guarnieri body. However, when such stained bodies are examined with care, one can see that they consist of granular material, that their contours are not always sharply defined, and that often, fine granular strands lead away from the main body, perhaps even connecting that body to another of similar character within the same cell.

The view that the Guarnieri body is intimately related to the Pasehen bodies, though it has been strongly contested by many writers on account of the inadequacy of the evidence advanced, has been held by various authors who too have observed in stained material that the Guarnieri body has a finely granular structure. Summarizing the evidence available, Topley and Wilson in their ' Principles of Bacteriology and Immunity " (1936) conclude that " the Guarnieri corpuscles probably represent actual intra-cellular micro-colonies of the virus ".

In view, therefore, of the observations on living cells, reported in this paper, there can be no longer valid reason to doubt that the Guarnieri body is simply a collection of vaccinal elementary bodies. It is, however, not a well-defined inclusion like the inclusion body of ectromelia or the Bollinger body of fowl-pox.

The study of cells showing the typical " ballooning colliquation " suggests that this is due to an increase of both the virus particles and the surrounding matrix at the expense of the original cell cytoplasm which largely disappears. The matrix appears to be less viscous than normal cell cytoplasm because the amplitude and frequency of the vibration of the virus particles are greater than is shown by granules and mitochondria in normal cells.

At first sight it would appear as if this increased fluidity could be explained as a simple change of state, the gel-like colloidal system of normal cytoplasm changing to the sol condition in the cell areas overrun by the virus bodies. But in view of the enlarged size of the " ballooned " cell the change to fluid state would seem, with more probability, to be due to disintegration of the cytoplasm with accompanying disturbance of osmotic conditions and consequent absorption of water. The type of cytoplasmic change—for so the formation of a matrix seems best regarded—which is induced in a cell is in some measure characteristic of the attacking virus. In psittacosis, at a certain stage in the development of a cell infection, Bland and Canti (1935) observed the virus bodies in vibratory movement somewhat similar to that which is exhibited by the virus bodies of vaccinia, but our own observations on the inclusion bodies of ectromelia indicate changes of a different character, as also do our (unpublished) observations on the Bollinger bodies of fowl-pox.

SUMMARY.

A method is described by which the living chorio-allantoic membrane may be examined microscopically by annular oblique incident illumination whilst it is still *in situ* in the egg, and with its vascular and other anatomical connections still undisturbed.

The method has been applied to the study of virus bodies within the living cells of the chorio-allantoic membrane of the duck egg infected with ectromelia and vaccinia viruses.

Observations on the nature of the ectromelia inclusion body are reported, and the formation of extra-cellular giant aggregates is described.

The presence and distribution of vaccinal elementary bodies within the living cell has been observed from early stages of cell infection. The elementary bodies, as they exist in the cell, are contained within a matrix of low viscosity and their partial release in " extrusion bodies " is described.

It is concluded that the vaccinal elementary body represents the only true virus structure which can be recognized in the living cell, and that the Guarnieri bodies, seen in stained preparations, correspond simply to localized irregular collections of elementary bodies, and do not necessarily represent all the virus in the cell.

Most of these investigations were carried out at the National Institute for Medical Research. I wish to express my gratitude to the Medical Research Council for their hospitality.

I also acknowledge my deep indebtedness to Sir Patrick Laidlaw for his constant guidance and encouragement in this work ; to Mr. J. E. Barnard for many favours ; and to Dr. W. J. Purdy for much help in the preparation of the drawings.

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PLATE I.

Infectious ectromelia in the chorio-allantoic membrane of the duck egg. Annular oblique incident illumination employed, except where otherwise stated. In the key the *shaded* areas indicate the positions of the more easily recognizable inclusion bodies and aggregates of free elementary bodies. The nuclei *N* are degenerate.

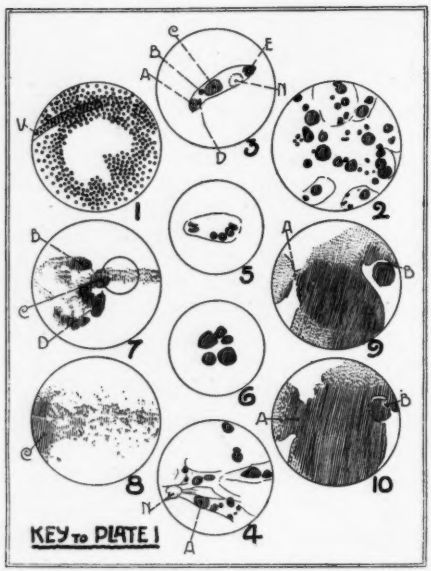


Fig. 1.—× 70. 60-hour nodule. Over the nodule all detail is lost in the general glare, but around it, corresponding to the dotted areas of the key, large numbers of invading leucocytes are seen, involving the vessel *V*.

Fig. 2.—× 450. Uppermost epidermal layer over the centre of a 48-hour lesion. The large number of inclusions is very conspicuous. Under the conditions which obtain in such situations much fine detail is inevitably lost in the general glare.

Fig. 3.—× 600. Fibroblast from the deeper layers of a 4-day lesion. There are 5 clearly recognizable inclusion bodies. Their general character and granular structure is seen in *C* and *E*. Inclusions *A*, *B* and *D* are not in sharpest focus.

Fig. 4.—× 450. Fibroblasts from 3-day lesion, showing inclusion bodies of various sizes. At *A* is an inclusion body adapted to the contour of the cell.

Fig. 5.—× 600. Epithelial cell from 3-day lesion, showing several inclusion bodies in sharp focus.

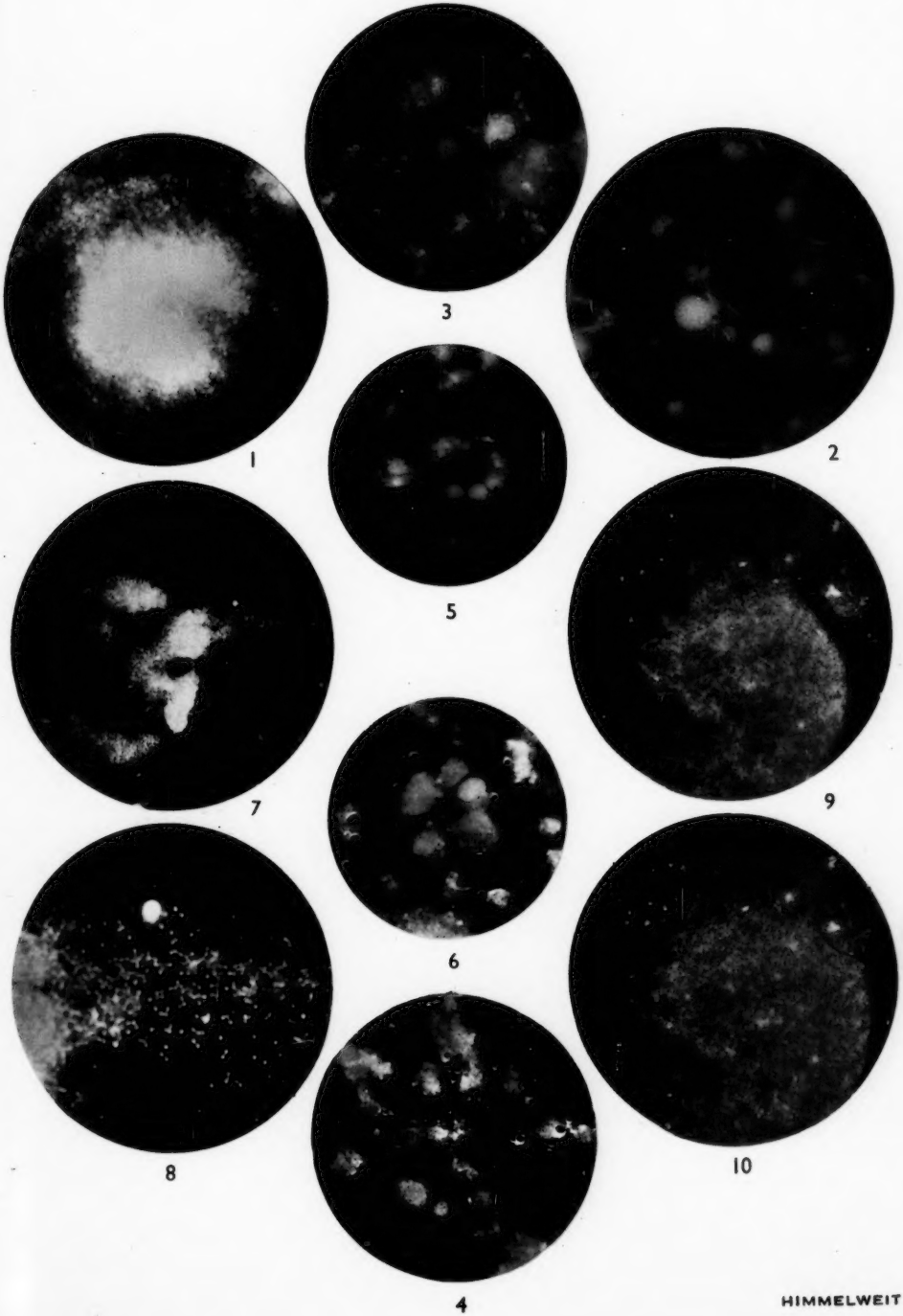
Fig. 6.—× 600. Group of inclusion bodies from a 5-day lesion. They are probably extra-cellular.

Fig. 7.—× 600. Inclusion bodies *B*, *C* and *D* from a 3-day lesion. Scrapings of the lesion were mounted in saline. Pressure on the coverslip has liberated streams of elementary bodies, demonstrating the soft and sticky nature of the matrix. Ordinary dark-ground method of illumination.

Fig. 8.—× 2200. Enlargement of part of Fig. 7—the part indicated by the small circle in the key—showing how the liberated elementary bodies tend to be arranged in pairs and short chains. Inclusion *C* corresponds to *C* in the previous picture.

Fig. 9.—× 500. Scraping from a 3-day lesion. Giant aggregate of elementary bodies, which has formed in the saline that constitutes the mounting medium. At *A* and *B* smaller aggregates are beginning to coalesce with the main mass. Ordinary dark-ground illumination.

Fig. 10.—× 500. Same field as shown in Fig. 9, but photographed 4 hours later. The process of coalescence has advanced. *A* and *B* are the same areas as have been indicated by these letters in Fig. 9.



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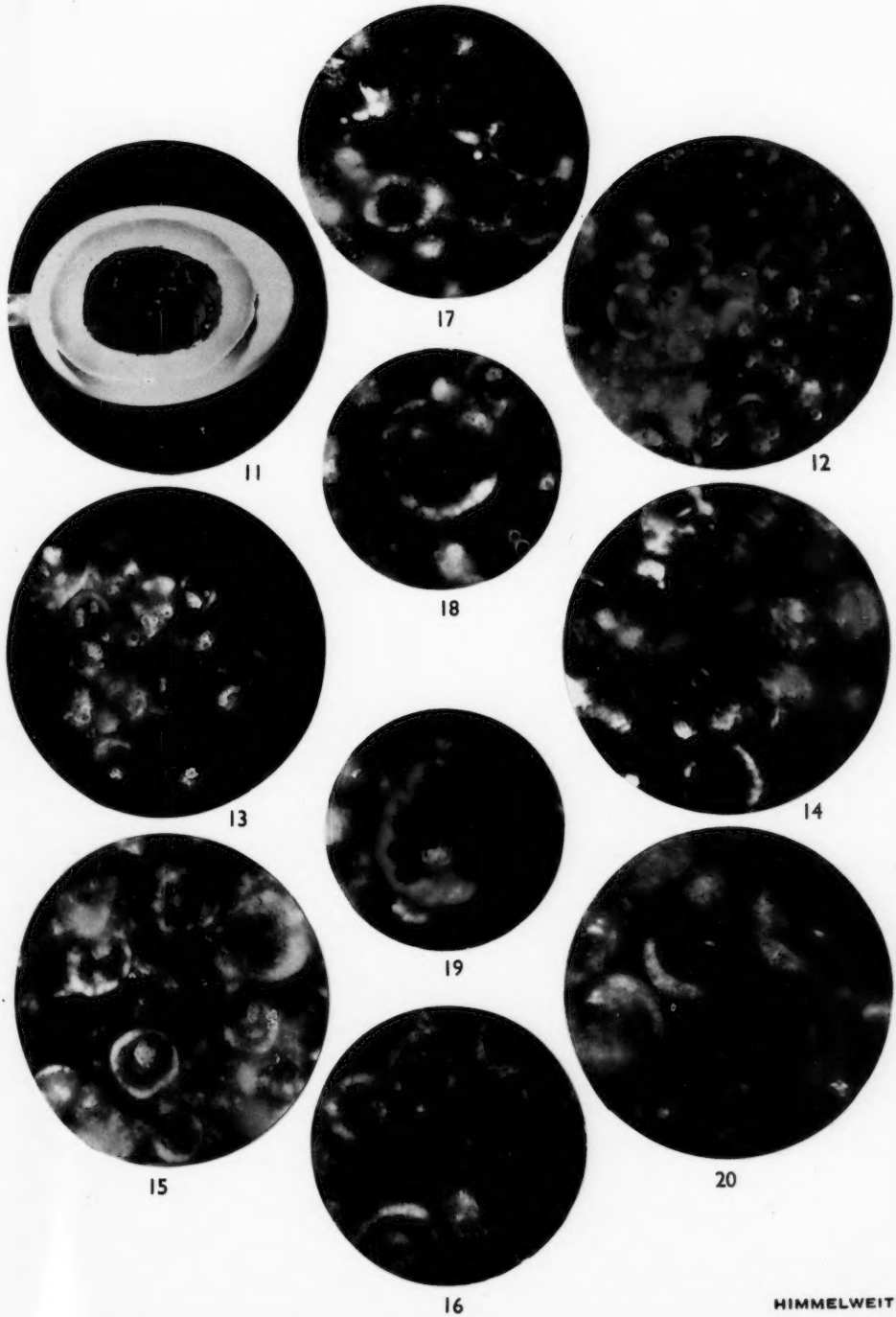
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PLATE II.

Vaccinal infection of the chorio-allantoic membrane of the duck egg. Oblique incident illumination used, except for Fig. 11. The areas which are *shaded* in the key indicate the more easily recognizable accumulations of elementary bodies. Where virus particles are still in rapid Brownian movement the virus masses have a characteristic milky appearance due to blurring of the photographic image. Most of the cells shown have become more or less swollen and rounded, as a result of "ballooning colliquation". In all areas *N* indicates the degenerated nucleus or nuclear remains and *E.B.* indicates a characteristic "extrusion body".

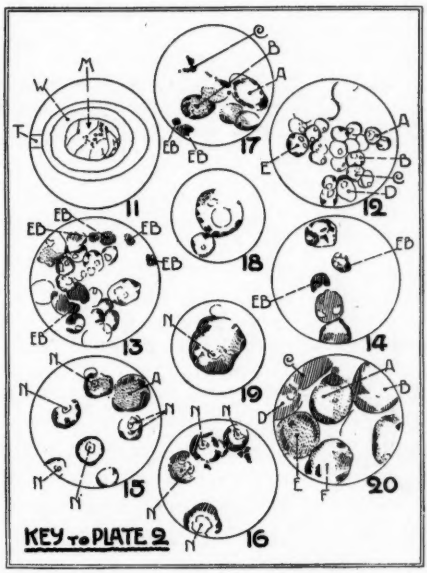

FIG. 11.—72-hour lesions. About 20 discreet nodules are seen indicated in the key by black dots. *M* indicates the chorio-allantoic membrane; *T* the tube through which the natural air-sac is inflated; and *W* the wax by which the coverslip is sealed to the egg-shell. The coverslip is not seen. It's edges have been cut away until they coincide with the outer limits of the wax.

FIG. 12.—× 450. 48-hour lesion. Early stages of cell infection. In cells *A*, *B* and *C* the virus is confined to a thin peripheral zone. *D* and *E* represent cells in a somewhat later stage of infection.

FIG. 13.—× 450. Cells at the edge of a 3-day lesion. They show advanced stages of cell-infection. Several "extrusion bodies", *E.B.* are present. The one towards the bottom of the photograph is in sharp focus; the others are in lower planes.

FIG. 14.—× 700. 3-day lesion. Two "extrusion bodies" are particularly well shown amongst the infected cells.

FIG. 15.—× 700. 4-day lesion. The surface of cell *A* is in sharp focus and the granularity of the sheet-like, superficial zone, invaded by the virus is evident. Granules are seen clearly because here vibration of virus particles had ceased. The granulation is rather coarse, however, and it is probable that in this case small aggregations of virus, rather than individual virus particles, are represented.

FIG. 16.—× 700. 4-day lesion. Advanced stage of cell-infection. Granularity attributable to virus particles is seen in parts where Brownian movement has ceased.

FIG. 17.—× 700. Cells on sloping edge of 4-day lesion. The high degree of contrast and consequent clarity of image presented by cells in this situation is illustrated. At *C* are well-defined intracellular virus accumulations, and two "extrusion bodies" are present, *E.B.*

FIG. 18.—× 700. Large "ballooned" cell showing the "beaded" arrangement of virus accumulations which is characteristic of a certain stage of cell-infection.

FIG. 19.—× 1100. "Ballooned" cell. The irregularity of the inner boundary of the virus accumulations is shown.

FIG. 20.—× 1100. 4-day lesion. "Ballooned" cells, showing appearances presented when Brownian vibration of virus particles has ceased. Diametral planes of cells *A* and *B* are focused upon, whilst in cells *C*, *D*, *E* and *F* it is the more superficial planes which are in sharpest focus. With regard to the very conspicuous granularity which illustrates conclusively the presence of virus bodies in these cells, the remarks made to Fig. 15 are here also applicable.

THE CHEMICAL COMPOSITION OF THE ACTIVE AGENT OF THE ROUS SARCOMA No. 1.

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In a previous communication it was shown that the removal of the greater part of the lipoid fraction of the Rous Sarcoma No. 1 produced no significant change in its infectivity (Pollard and Amies, 1937). Extraction of the dry material with lipoid solvents did not remove the tumour-producing activity, nor could any activity be found in the lipoid extracts after filtration through Seitz or Berkefeld (W) filters. These results were contrary to the findings of Jobling and Sproul (1936) and of Jobling, Sproul and Stevens (1937), who suggested that the tumour-producing agent was essentially lipoid in nature; but were in agreement with the experiments of Fraenkel and Mawson (1937) and of Levine and Baumann (1937), who were unable to demonstrate any activity in filtered or centrifuged extracts. The investigation has been continued and chemical analysis of the active fraction of the tumour has been undertaken.

A method was required by which quantities of the active material sufficient for anaylsis could be obtained free from the inactive constituents of the tissue. Methods of purification using precipitation and absorption give the material in the form of dilute suspensions, which are not directly suitable for chemical analysis. The work of McIntosh (1935), Ledingham and Gye (1935), Elford and Andrewes (1935) and of Amies (1937) has shown that the tumour agent can be thrown down by high-speed centrifugation, and Amies (1937) and Claude (1937) have described the preparation of purified suspensions by fractional centrifugation.

To obtain quantities of material sufficient for analysis requires the centrifugation of larger volumes of tumour extract than are conveniently taken by the usual types of bucket centrifuge. The Sharples centrifuge has accordingly been applied in the experiments to be described. A machine of this type was utilized by Schlesinger (1936) for the deposition of bacteriophage, the material being collected on a layer of agar lining the bowl of the machine. The presence of agar was undesirable in the present case and the tumour extracts were, therefore, centrifuged directly in the metal bowl. The coherent deposit formed on the surface of the metal could readily be collected and dispersed in a suitable medium. This deposit contained the greater part of the tumour-producing

activity of the original extract, as shown by the results given in Table I. The re-suspended deposit was then purified by fractional centrifugation as in the typical experiment described below.

Centrifugation of tumour extract.—Fresh tumour tissue was minced, frozen and thawed six times to break up the cells and then ground with sand. A 5 per cent. suspension of this tumour pulp was prepared in physiological saline containing hydrogen cyanide (1/10,000) and buffered to pH 7·4. This was centrifuged at 3500 r.p.m. for 40 min. to remove the greater part of the tissue debris. The clarified extract was carefully withdrawn and centrifuged in portions of 300 ml. in the batch bowl of the Sharples centrifuge for three-quarters of an hour at 36,000 r.p.m., which gave a centrifugal force of 33,000 times gravity. The bowl and its contents were cooled to a temperature of 5° C. before commencing the run, so that the final temperature of the liquid was not higher than about 25° C. Before the bowl was opened at the end of each run, any grease or oil on the outside was washed off with benzene and care was taken that no such contamination entered the bowl at any time. The deposits resulting from several such centrifugations were rubbed off the metal surface and dispersed in distilled water or M/200 phosphate buffer of pH 7·4. The suspension so obtained was again centrifuged at 3500 r.p.m. for half to three-quarters of an hour, the supernatant liquid collected and the considerable deposit of fine tissue debris was re-suspended in the same medium and centrifuged at low speed as before. The supernatant fluids from several such washings were combined and again centrifuged at low speed and any deposit which formed was discarded. A liquid with a bluish-white opalescence was obtained which formed no deposit on standing for several days at 3–5° C. Microscopic examination with dark-ground illumination showed a homogeneous dispersion of small particles of approximately the same optical appearance.

Inoculation.—The activity of the purified deposit was compared with that of the original tumour extract and that of the supernatant fluid from the Sharples centrifuge, by re-constituting an aliquot portion to the original volume and preparing from each liquid serial dilutions in saline. The dilutions were injected in volumes of 1 ml. into the breast and leg muscles of 10-week-old chickens (cross-bred White Leghorn and Plymouth Rock). Comparable sites on different sides of the same bird were used to compare the activities of two given solutions to minimize errors arising from variations in the individual

TABLE I.—*Deposition of Agent of Rous Sarcoma No. 1 by the Sharples Centrifuge.*

Experiment number.	Titre of tumour extract.	Titre of supernatant.	Titre of reconstituted deposit.	Dry weight of deposit in mg. in a minimal tumour-producing dose.
1	1/1000	1/1000	1/10,000	Not tested.
2	1/1000	1/1000	1/5000	$4 \cdot 10^{-6}$
3	1/1000	1/100	1/1000	$2 \cdot 10^{-4}$
4	1/5000	1/10	1/1000	$5 \cdot 10^{-5}$
5	Not tested	1/10	1/1000	Not tested
6	,,	Not tested	1/10,000	$1 \cdot 10^{-5}$
7	,,	,,	1/10,000	$2 \cdot 10^{-6}$

susceptibility. In the majority of the experiments the inoculations were carried out in triplicate. The results are shown in Table I, where the amount of material required to produce a tumour is given as the limiting dilution and as the approximate dry weight in mg. of the minimal tumour-producing dose of the purified deposit.

Further purification and drying.—The bulk of the purified suspension was freed from the inorganic salts in solution by centrifugation for 1 hour in a Swedish angle centrifuge at 3500 r.p.m. This gave almost complete sedimentation of the active material, the deposit appearing as a white translucent gel which readily dispersed in distilled water to yield an opalescent fluid as before. This process of sedimentation and dispersion was repeated several times and the deposit was finally taken up in a small volume of distilled water. The resultant thick suspension was dried from the frozen state *in vacuo* and yielded a cream-coloured voluminous mass which could readily be manipulated. About 20–30 mg. of material could be obtained from 100 g. of fresh tumour.

Analysis.—After drying to constant weight at 110° C., the material was exhaustively extracted with boiling benzene, whereby the lipoid material which constitutes from 30–40 per cent. of the dry weight of the deposit was removed. The residue contained about 15·5 per cent. of nitrogen (ash-free) as determined by micro-Kjeldahl estimation. A variable amount of ash was found in the preparations, which was derived in part from traces of inorganic matter adsorbed on the material. Some typical analyses are given in Table II.

TABLE II.—*Composition of Purified Suspensions of Agent of Rous Sarcoma No.* 1.

Experiment number.	Lipoid, per cent.	Ash, per cent.	Nitrogen, per cent. (ash- and lipoid-free).
1	46	5·8	15·1
2	40	3·7	15·5
3	40	2·0	15·3
4	48	Not estimated	Not estimated
5	40	,,	,,
6	35	,,	,,

To determine whether the activity of the deposit depended on the presence of this lipoid material, methods by which it could be removed were investigated. Preparations of the tumour agent obtained by fractional centrifugation were dried from the frozen state and extracted with benzene at room temperature in an atmosphere of nitrogen under diminished pressure. Although dried preparations still showed tumour-producing activity, dried and extracted preparations were completely inactive. The considerable diminution of activity which took place on freezing and drying occurred largely during the process of freezing. For although the tumour tissue may be frozen and thawed without loss in activity, the purified preparations showed a distinct fall in activity when treated in this manner. As shown in Table III, the activity of a preparation was reduced to about one tenth of its previous value by freezing and thawing, while subsequent drying reduced the activity still further. The process of freezing and thawing was accompanied by a change in the physical

state of the material, the white opalescent fluid being converted into a suspension of visible aggregates. Extraction of purified suspensions with ether at 0° C. caused complete inactivation, and crude tumour extracts are similarly inactivated. On the other hand, vaccinia virus which in many ways behaves similarly to the tumour agent, has been shown by Craigie (1934) to retain its activity in the presence of ether. Treatment of the purified tumour agent with mixtures of alcohol and ether at lower temperatures (— 15 to — 5° C.) also yielded inactive material.

TABLE III.—*Effect of Freezing and Thawing and of Desiccation on Purified Suspensions of the Tumour Agent.*

Experiment number.	Titre of control.	Titre of frozen and thawed suspension.	Titre of reconstituted suspension after freezing and drying.
1	1/1000	Not tested	1/100
2	1/1000	,,	1/10
3	1/10,000	,,	1/100
4	1/10,000	1/100	1/10
5	1/100	1/10	Not tested
6	1/1000	1/100	1/1

In all cases the volume of the inoculum was 1 ml.

In the earlier experiments, solvent extraction of tumour desiccates had been found to cause no significant loss of tumour-producing activity and this method was, therefore, adopted to obtain preparations of the tumour agent with a lower lipoid content. Extraction of desiccated tumour tissue with anhydrous solvents, including alcohol, ether, benzene, light petroleum or chloroform in a Soxhlet apparatus in nitrogen under reduced pressure yielded tumour residues still capable of producing tumours; and from which the active material could still be separated by fractional centrifugation. Preparations purified by these methods were now found to contain much smaller quantities of extractable lipoid material, but as shown in Table IV, they showed tumour-producing activities of the same order as before. When alcohol was used as a solvent, the activity of the residual desiccate and of the purified material appeared to depend on the temperature at which the extraction was carried out rather than on the amount of lipoid removed. The effect may be attributed to a temperature inactivation or denaturation by the solvent rather than to the removal of an essential constituent. The results given in Table IV indicate no relation between the activity of a given preparation and its lipoid content, for in two of the most active preparations the amount of extractable lipoid has the lowest values. The nitrogen content of the residue (ash- and lipoid-free) was about 16·5 per cent. in the majority of cases and only a trace of carbohydrate could be detected after acid hydrolysis. According to Hughes, Parker and Rivers (1935), the elementary bodies of vaccinia contain about 8·5 per cent. of lipoid material, a value which is comparable with that found in the lipoid-extracted preparations of the tumour agent. The nitrogen content of the vaccinia residue (ash- and moisture free) was about 15·5 per cent.,

TABLE IV.—*The Effect of Lipoid Solvents upon Desiccates of the Rous Sarcoma No. 1.*

Experiment number.	Solvent (anhydrous).	Time of extraction (hours).	Temp., °C.	Lipoid extracted, per cent.	Titre of extracted desiccate.	Titre of purified tumour agent.	Mg. deposit in minimal tumour-producing dose.	Analysis of purified tumour agent.		
								Lipoid, per cent.	Nitrogen, per cent. (lipoid and ash-free).	Ash, per cent.
1	Benzene	14	20	Not estimated	1/1000	1/1000	$5 \cdot 10^{-5}$	22·0	15·0	5·7
2	Ether	14	20	6·0	1/1000	1/1000	$1 \cdot 10^{-4}$	15·0	15·2	5·0
3	(Alcohol 7, Ether 11)		20	11·0	1/1000	1/1000	$2 \cdot 10^{-5}$	6·0	Not estimated	Not estimated
4	(Alcohol 8, Benzene 4, Ether 2)		20	12·5	1/10	1/10	$5 \cdot 10^{-4}$	12·5	,,	,,
5	(Alcohol 8, Benzene 2, Ether 1)		25–30	10·0	1/500	1/10	$2 \cdot 10^{-3}$	9·7	15·3	7·8
6	(Alcohol 9, Benzene 2, Ether 1)		30–37	11·0	Inactive	Inactive	..	20·0	16·8	7·0
7	Petroleum + chloroform	11	20–35	9·0	Not tested	1/100	$5 \cdot 10^{-5}$	6·5	16·7	6·2
8	Petroleum + chloroform	15	20–35	7·0	1/1000	1/10	$1 \cdot 10^{-3}$	11·0	16·4	3·9
9	(Petroleum + chloroform, 8; alcohol, 1)		20–35	8·0	1/1000	1/1	$1 \cdot 10^{-3}$	13·0	16·5	5·2

Solvents in brackets were used in sequence.

which is of the same order as the values found in preparations from fresh tumour material, but lower than the values found in lipoid extracted preparations. The higher nitrogen figures in the latter preparations may be due to a more complete removal of the non-protein constituents in the preliminary treatment.

Purified preparations of the tumour agent therefore consist mainly of protein with a high nitrogen content, with a small amount of lipoid material which may be an essential constituent of an organized system, or may on the other hand, be merely an impurity, which is not completely removed in the earlier stages of the preparation.

SUMMARY.

The tumour-producing agent of the Rous sarcoma No. 1 can be deposited from cell-free extracts by means of the Sharples centrifuge. The re-suspended deposit can then be further purified by the method of fractional centrifugation. The product obtained consists mainly of protein with a variable amount of lipoid material. Preparations from lipoid-extracted tumour desiccates contain much smaller amounts of lipoid, but still retain their tumour-producing activity.

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THE FORMAMIDE METHOD FOR THE EXTRACTION OF POLYSACCHARIDES FROM HÆMOLYTIC STREPTOCOCCI.

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THE study of the specific bacterial polysaccharides has become increasingly prominent in the problem of the systematic classification of bacteria. The importance of polysaccharides in this connection was first realized when the specific substance discovered in pneumococcus culture filtrates by Dochez and Avery (1917) was isolated and shown to be a polysaccharide by Heidelberger and Avery (1923). Since then it has been found that each type of pneumococcus is characterized by a definite type-specific polysaccharide.

More recently similar polysaccharides have been prepared from a large number of bacterial species.

In a series of papers on the hæmolytic streptococcus, Lancefield (1933) has differentiated this organism serologically into a number of groups. Each group has definite cultural and biochemical characteristics, and owes its serological specificity to a polysaccharide "C" substance present in appropriate extracts, and demonstrable by the precipitation reaction.

It has been shown by Lancefield and Hare (1935) that practically all the serious puerperal infections caused by hæmolytic streptococci are due to Group A organisms. It is of the utmost importance, therefore, in the prophylaxis of puerperal fever to determine quickly whether the streptococci so frequently present in the noses and throats of attendants at confinements belong to the dangerous Group A.

Lancefield obtains her extracts by Porges' method—heating the deposited cocci with N/20 HCl. The method is a good one, but has certain drawbacks. The extracts contain non-group-specific substances which may give cross-reactions in the precipitation test if the antisera employed contain precipitating antibodies to fractions other than the C substance. Laboratories making their own grouping sera may sometimes have only such imperfect samples available.

Secondly the Lancefield method is an uneconomical one, since the extraction process leaves a large residue of undissolved bacteria. For this reason (unless the process is repeated several times) it is necessary to grow relatively large lots of broth culture (at least 50 c.c.) and to have available an expensive large centrifuge. It was felt that this difficulty could be overcome—and the method

thereby made available for small laboratories—if a solvent could be found which would dissolve the bacteria completely. With the method here described 5 c.c. or less of broth culture is sufficient for determining the group reaction of any strain, and cross-reacting substances are, to a large extent, destroyed or removed during the process of extraction, so that satisfactory results may be obtained with low-grade sera.

Method of Extraction.

A search was made for an organic solvent which would dissolve the bacteria completely and so give a good yield of extract quickly. Some of the solvents employed, *e. g.* warm phenol, dissolved the bacteria, but on diluting with water the bacterial substance was precipitated and very little soluble material was obtained. Experiments with other solvents that might be expected to dissolve proteins, such as lactic acid, benzaldehyde, ethylene glycol, paraldehyde, pyridine, glacial acetic acid and saturated aqueous urea were not successful. It appeared that the polysaccharide was in combination with other cellular substances, and that it was necessary to break this down before an extract could be obtained.

Formamide was found to be a suitable solvent. It is a colourless viscous liquid, boiling at 195° C. It dissolves a large variety of substances, and acts in the present instance by breaking down the complex protein substances of which bacteria are largely composed. Cold formamide does not dissolve streptococci or extract the group specific substance from them, but boiling formamide dissolves the bacteria to give a clear solution in 1 minute. At 150° C., solution of the bacteria is practically complete in 5 minutes; at lower temperatures the action is much slower. The polysaccharide is quite stable in hot formamide solution, being undiminished in titre after 1 hour at 150° C.

Details of method.—5 c.c. of an 18-hour glucose broth culture are spun down in the centrifuge and the supernatant is removed as completely as possible. 100 c.mm. of formamide are added to the residue, and the tube shaken and placed in an oil bath at 150° C. for 15 minutes. The tube is allowed to cool, and 250 c.mm. of acid alcohol* are added, mixed, and the precipitate removed by centrifuging.

The acid alcohol throws down bacterial *débris* and partially broken-down proteins, while keeping the group antigens† in solution. In those groups so far examined where the type-specific antigen is a polysaccharide, this is also precipitated.

The supernatant is drawn off by pipette, placed in a small tube, and 500 c.mm. of acetone added. The tube is shaken, the precipitate spun down and the supernatant discarded.

The acetone precipitate is very small in amount, but it contains practically all the group antigen.

* A mixture of 95 parts of anhydrous alcohol with 5 parts of 2 N (1 part concentrated acid with 4 parts water) hydrochloric acid.

† The group specific polysaccharide is referred to in this paper as an "antigen" for convenience. It is not a true antigen, for it does not give rise to antibodies on injection.

One c.c. of saline and a drop of phenol red indicator are added to the precipitate, and the whole neutralized with a trace of sodium carbonate. This solution is used for the test.

Since the antigen is isolated in solid form, the final volume may be varied at will. If the growth in the broth culture has been poor, the extract may be diluted to much less than 1 c.c. if desired.

The acetone supernatant contains the simpler protein breakdown products, and if excess acetone is added to it a large amount of inert material is thrown down.

A purer extract is obtained if the acetone precipitate is stirred with saline before neutralizing. A part does not dissolve, and may be spun down. The supernatant is then neutralized and used for the test. This extra step may be desirable if the sera used contain much anti-nucleoprotein antibody.

The simplest method of extraction is to dilute the formamide solution of the bacteria with saline, neutralize, spin down the residue, and use the supernatant for testing. The extract, however is not as satisfactory as that prepared by the above method.

The temperature of extraction is not critical, since good extracts may be made by boiling the bacteria with formamide for a few minutes. The temperature, however, should not be below 150° C., or the bacteria dissolve very slowly.

Since the type-specific M substance of group A hæmolytic streptococci is destroyed by formamide in common with other proteins, the extracts are of no use for the typing of group A strains. It is this destruction of protein by formamide which helps to make the extracts more strictly group-specific.

Group antigens have been extracted and partly purified from all the known groups of hæmolytic streptococci except group E, no strain from this group being available at the time. They vary in their ease of precipitation with alcohol and acetone, and the above method is a compromise to make the method suitable for all groups.

Testing.

The extracts may be tested by the precipitation reaction in any of the usual ways, by mixing with the serum on a macro- or micro-scale, or by layering.

When different fractions were assayed for antigen in a later part of the work, they were tested at dilutions which increased fivefold, *e. g.* at 1 in 5000, 25,000, 125,000 and 625,000, and the reactions with the antiserum noted. This method does not show up small differences between fractions, but a method of separation is of no use unless it divides the material up into parts of widely different antigen content.

The nature of the precipitate varies with the proportions of antigen and antibody. When mixed in optimal proportions an even opalescence quickly develops, which, in an hour or two, settles to a translucent mass, like a piece of quartz. As the proportion of antigen is increased, the precipitate becomes so transparent and glassy that it is difficult to see, and a sufficient excess of antigen inhibits precipitation altogether. With the antiserum in excess the precipitates become more flocculent.

Extracts made by the formamide method are so strong that this inhibition of precipitation is a matter of practical importance in the routine grouping of streptococci, especially if the sera used are weak in antibody. A negative result should not be accepted until the test has been repeated with a considerably diluted extract.

Results of Extractions by the Routine Method.

Before the formamide method was accepted for routine use it was tested on 44 strains of hæmolytic streptococcus previously grouped by the Lancefield method. Since then the method has been tried out in these laboratories for two years, and no objection to its use has been forthcoming. It has more than once produced a group-specific extract from a strain when the Lancefield method has failed to do so.

In all the following experiments 5 c.c. of broth culture were used:

Group A.—Twenty-five group A strains were tested. All gave good reactions with the antigen dissolved, as described above, in 1 c.c., and showed no cross-reactions at all with anti-B, C, F or G sera. The first 12 extracts were tested at several dilutions. In 7 of these the antigen was dissolved in 0·2, c.c. and the extracts failed to give a precipitin test owing to excess antigen. All reacted well when diluted to 1 c.c. or to 5 c.c., and 6 showed a definite reaction when diluted to 25 c.c.

Group B.—Six group B strains were tested. All extracts gave good reactions when dissolved in 1 c.c. or 5 c.c. of saline. With the antigens dissolved in 0·2 c.c. the tests were much weaker, the excess antigen partly redissolving the precipitates. The alcohol precipitates also reacted strongly, and a little active material was present in the acetone filtrates. The main extracts gave no crossing with A, C or G sera, while the alcohol precipitate gave various small fluffy cross-reactions.

Group C.—Four strains were extracted. These showed inhibition of precipitation by excess antigen when the antigens were dissolved in 0·2 c.c., and reacted well at a dilution of 1 c.c. and 5 c.c., one extract reacting at a volume of 25 c.c. No crossing occurred against anti-A, B or G sera. The alcohol precipitates contained reactive substance, and also gave small cross-reactions with heterologous sera. The acetone filtrate contained a little group antigen.

Group F.—Extracts from two strains gave satisfactory reactions when dissolved in 1 c.c.

Group G.—Four strains yielded extracts which gave good reactions at dilutions of 1 c.c. and 5 c.c. with no cross-reactions. The alcohol precipitate and the acetone filtrate both contained some group antigen, and also crossed with heterologous sera.

Group H.—Three strains were extracted. Extracts from two of them reacted at a dilution of 1 c.c., but not at 5 c.c., while the other strain (Smith), failed to give a group reactive extract. All three strains when extracted by Lancefield's method gave opaque precipitates with anti-H serum, while the formamide extracts from the first two yielded clear glassy precipitates.

It is possible that the Smith strain was not a group H strain, and that the reaction obtained with the Lancefield extract was due to some non-group specific substance. When formamide extracts are used the true group reaction always produces a clear glassy precipitate when extract and serum are mixed in the appropriate proportions. Lancefield extracts, on the other hand, often give opaque or fluffy precipitates which may not always be due to a group specific reaction.

Larger Scale Extractions.

Preliminary experiments were made on the purification of the formamide extracts prepared from larger quantities of bacteria.

Group A.

The solution of streptococci in formamide was precipitated with 10 volumes of acetone, dissolved in water, and neutralized with sodium carbonate. Many of the earlier attempts at fractionation were a failure, that is, they failed to concentrate the antigen into any one fraction. For conciseness a list of these methods is given below, with no details of procedure.

Fractional precipitation with trichloracetic acid, ammonium sulphate, phosphotungstic acid, tannic acid, zinc acetate, cadmium chloride, ethyl alcohol in presence of sodium acetate. Fractional iso-electric precipitation. Extraction of the solid with ethylene glycol or phenol-alcohol mixtures. Adsorption on alumina or ferric hydroxide.

The usefulness of acidified alcohol and acetone in the purification was then discovered, and the following method of purification was adopted :

The bacteria were spun down, dried in acetone and heated for 20 minutes with 15 volumes of formamide at 150° C. After cooling, $2\frac{1}{2}$ volumes of acidified alcohol (containing 5 p.c. of 2 N. hydrochloric acid) were added, the precipitate filtered off, and re-extracted twice with small quantities of 70 p.c. alcohol. The combined alcohol extracts were mixed with an equal volume of acetone, and the precipitate spun down. Examination showed that this precipitate contained more than 99 p.c. of the extracted antigen. The precipitate was dissolved in a small quantity of water. A part remained undissolved, and small amounts of acid were added till precipitation was maximal. This insoluble fraction was probably largely nucleoprotein.

A further purification was effected by fractional precipitation with acidified acetone.* Although the antigen was precipitated by one volume of acetone from alcoholic solution, 5 volumes of acetone were required to precipitate it from aqueous solution. When only 2 volumes of acetone had been added, a fraction containing only a small amount of antigen was brought down. The material was thus separated into parts which had been precipitated with 2, 5 and 10 volumes of acetone respectively. Although most of the antigen was present in the 5-volume precipitate, both the other fractions contained appreciable amounts, which could be removed by reworking the fractions.

A test showed the 5-volume precipitate to be more than 100 times as pure as the 2-volume precipitate. A typical experiment gave the following figures :

Volume of broth	44 litres
Weight of dried streptococci	41 g.
Weight of 2-volume precipitate	0·24 g.
Weight of 5-volume precipitate	0·54 ,,

The polysaccharide could be further purified by pyridine. By extracting with small amounts of 80 p.c. pyridine three times, a gummy residue containing only traces of group-specific material was left. In a typical experiment, 0·32 g. was insoluble in 80 p.c. pyridine, and 0·48 g. was soluble.

* Acetone containing 5 p.c. of 2 N. hydrochloric acid.

The pyridine-soluble part was precipitated with excess acetone. It could be separated into fractions soluble and insoluble in 90 p.c. pyridine, but the amount of purification effected was hardly worth the labour involved.

None of the soluble fraction was soluble in anhydrous pyridine; only a small part dissolved in 95 p.c., while nearly all was soluble in 90 p.c. pyridine, leaving a slightly less pure residue. The fractions obtained by repeated extraction with 95 p.c. pyridine were not widely different in antigen content.

Attempts to purify the polysaccharide further have failed so far. A brief *résumé* of them is given below. The pyridine-soluble fraction was used in these tests.

Glacial acetic acid.—The material was completely soluble in 95 p.c. acetic acid, while only a small amount was soluble in the glacial acid. Four volumes of glacial acid were added to a solution in 95 p.c. acetic (final concentration 99 p.c.). At this concentration about half was soluble, but both soluble and insoluble parts had about the same antigen content.

Methyl alcohol.—The solubility in acidified methyl alcohol was about the same as that in acetic acid. About one-third by weight was soluble in 99 p.c. methyl alcohol (containing 1 p.c. of 2 N. hydrochloric acid). This fraction was slightly more active than the insoluble one.

With 95 p.c. methyl alcohol containing 5 p.c. of 2 N. acid about 20 p.c. was insoluble, but again the two fractions were of much the same activity.

Continued extraction with anhydrous methyl alcohol dissolved about 20 p.c., but no separation of activity resulted.

Cyclohexanol dissolved a small fraction but without selection of antigen.

Isopropyl alcohol.—The material was insoluble in 100 p.c., soluble in 50 p.c. containing 0·1 N. hydrochloric acid. At 70 p.c. strength 70 p.c. was insoluble, and this fraction seemed a little more active than the soluble part.

Charcoal adsorption.—Charcoal removes at least 99 p.c. of the antigen from a 0·1 p.c. aqueous solution, whether the solution is neutral or slightly acid. A large part of the antigen could be removed from the charcoal by extracting it with 90 p.c. pyridine. 75 p.c. alcohol and aqueous lithium thiocyanate extracted less of the antigen, while 75 p.c. acetone was less successful still. No purification was effected by this adsorption on charcoal and elution with 90 p.c. pyridine, and a considerable loss of antigen occurred.

Since many attempts at purification had failed, it was thought advisable to see how much polysaccharide was present in the preparation.

The pyridine-soluble fraction was heated for 15 minutes at 100° C. with N. sulphuric acid, neutralized, and the reducing sugar determined by the Folin-Wu method against a glucose standard. The reducing sugar produced by the hydrolysis of the polysaccharide was more strongly reducing than glucose, for 4 mg. of the polysaccharide after hydrolysis had the reducing power of 6·4 mg. of glucose. In another experiment, 5 mg. of polysaccharide after hydrolysis had the reducing power of 8 mg. of glucose.

This showed that the preparation was all polysaccharide, whether or no it was all antigen. It still gave a faint biuret test, but this seemed due to impurity, for after heating with formamide at 200° C. for 30 minutes the biuret test was negative, while the specific activity of the material seemed undiminished. After this reheating the antigen was not completely precipitated by 5 volumes of acetone. It is possible that the antigen is only a small part of the polysaccharide portion of the bacteria.

In the present fractionation organic solvents have been used, to lessen the chances of adsorption of antigen on to inactive precipitates. Many other reagents have been tried, but no fractionation has resulted. Picric acid,

flavianic acid, mono-, and di-, nitro phenylhydrazines, lead acetate, various heavy metal salts, brucine, quinine, semicarbazide, have all so far failed to be of use. The preparation of definite chemical derivatives of the polysaccharides or breaking them down without hurting the antigen may be necessary steps before further purification is possible.

Group B.

The crude formamide extracts from group B strains gave small cross-precipitations with antisera from all other groups. The substance responsible for this cross-reaction was destroyed by trypsin, was insoluble in slightly acid water, and was precipitated from aqueous solution by 3 volumes of acid alcohol. The greater part of the group-specific substance remained dissolved in the alcohol, but the precipitate contained some adsorbed antigen, which was removed when the precipitate was dissolved and reprecipitated. The alcohol precipitate gave a strong reaction with the antiserum from the homologous type, and probably contained the type antigen.

The fractionation of the alcohol-soluble part with acetone from aqueous solution was not so successful as with group A, as the antigen tended to spread over all fractions, even after they were re-worked. All the antigen was precipitated by 5 volumes of acidified acetone, but the fractions brought down with less acetone contained much reactive material.

The antigen was soluble in 75 p.c. pyridine, leaving a nearly inactive insoluble fraction.

Group C.

When 3 volumes of acid alcohol were added to an aqueous solution of the crude formamide extract, the precipitate contained non-specific material, while the soluble part contained the group antigen. A fraction of this precipitate was soluble in slightly acidified water, and this contained no antigen. The antigen was soluble in 75 p.c. pyridine, leaving a precipitate which gave only non-specific reactions.

Group D.

Group D extracts did not give very satisfactory results, largely because the only group D antiserum available at that time was poor in group antibody. Extracts from two strains heterologous in type to the testing serum gave very weak reactions. An extract from the homologous type yielded a strong reaction, and since the substance responsible for this reaction was precipitated by 3 volumes of acid alcohol, the reaction was probably a type-specific one.

Group F.

Extracts from a group F strain (H 19 a) reacted with group F antisera to give glassy precipitates when diluted to approximately the volume of the original culture. When the aqueous solution of the crude extract was precipitated with 3 volumes of acid alcohol, the precipitate reacted only with the

homologous antiserum, and so contained the type antigen. The alcohol-soluble part, in conformity with other groups, reacted with all anti-F sera, thus containing the group antigen.

An aqueous solution of the latter fractionated with acidified acetone gave a relatively inert precipitate with 2 volumes, but practically all the group activity was brought down with 3 volumes.

Another strain (H 19 c) similarly yielded the group antigen in the alcohol-soluble fraction. When the aqueous solution was fractionated with acid acetone, the antigen was spread over the various fractions, but after re-working, the 1-volume precipitate was comparatively inert, while $3\frac{1}{2}$ volumes brought down the bulk of the activity. The slight difference in the properties of the group antigen prepared from these two strains may be due to impurities present, or the antigen might not be identical in the two strains.

A fractionation with pyridine was not possible, as practically all the material was soluble in this solvent.

In this group, the antigen-antibody precipitates were very readily re-dissolved by excess antigen.

Group G.

The strain extracted was heterologous in type to the serum used. The 3-volume acidified alcohol precipitate contained very little group antigen. The alcohol-soluble fraction dissolved in water gave with 3 volumes of acidified acetone an inactive precipitate, while 5 volumes precipitated the antigen. As before, these fractions were dissolved and reprecipitated to make the separation complete.

Group H.

The group H extract from the homologous type reacted with the serum when dissolved in a volume up to half that of the original culture. The 3-volume alcohol filtrate contained most of the reactive substance, but the precipitate also gave a marked reaction. Extracts would have to be made from a heterologous type before it could be decided whether this latter reaction was a type-specific one, or was due to group antigen contained in the precipitate. When pyridine was added to the trypsinized extract, precipitates were obtained at 60 p.c. and 90 p.c. strengths. These precipitates contained the bulk of the group reactive material. The antigen in the pyridine-insoluble fraction was brought down from aqueous solution by 4 volumes of acidified acetone.

Group K.

The strain and serum used were of the homologous type. When an aqueous solution of the extract was precipitated with 3 volumes of acid alcohol, most of the reactive substance remained in the solution. The trypsinized material, as with group H, gave precipitates at 60 p.c. and 90 p.c. pyridine, but unlike that group, the group antigen was contained in the pyridine-soluble part. The antigen was precipitated from aqueous solution by 3 volumes of acid acetone.

Incidental Results.

Antigenicity of group A polysaccharide.

No demonstrable antibodies were produced when either the pyridine-soluble fraction from group A hæmolytic streptococci or its antigen-antibody precipitate were injected into rabbits.

The pyridine-soluble fraction up to a dilution of 1 in 625,000 gave a precipitate with an equal volume of group A serum. At concentrations of 1 in 3000, or stronger, precipitation was inhibited, while the reaction seemed maximal at 1 in 8000, and it was the precipitate from this mixture which was used for injection. Two rabbits were injected subcutaneously over 1 month, receiving in all the precipitate from 24 c.c. and 7 c.c. of serum respectively.

Cross-reactions.

It was stated above that the routine extracts obtained by the formamide method gave much less cross-reaction than those from Lancefield extracts from the same strain. This is illustrated in Table I, which shows the reaction of both types of extract against poor quality sera. These were old experimental sera which owing to long courses of inoculation contained many species specific antibodies. They were, of course, not representative of the antisera used for grouping at these laboratories, but were useful in showing the greater purity of the formamide extracts.

Extraction of pneumococci.

Formamide dissolved pneumococci produced type-specific extracts. 5 mg. of dried bacteria from each of types I, II and III were boiled for 1 minute with formamide, precipitated with acetone, and the precipitate dissolved in saline. Dissolved in 125 c.c., all three extracts gave good immediate precipitation reactions in the cold with the homologous serum, and none with the heterologous. When diluted to 1 litre they still gave a precipitation reaction after 1 hour at 37° C.

TABLE I.

Serum.	Lancefield extracts from—				Formamide extracts from—			
	Group A.	Group B.	Group C.	Group G.	Group A.	Group B.	Group C.	Group G.
Group C (X_3) .	+	+−	++	++	−	−	+	+−
„ G (XI_5) .	+−	+	+	++	−	−	−	+[18]+
„ C (XXVII)	+−	−	++	++	−	−	+++	−
„ A ($XIII_2$) .	++	++	+	+	++	−	−	−
„ G (XXI) .	++	+	+	++	+	−	+	+++

DISCUSSION.

This investigation of the principal groups of hæmolytic streptococci shows that the formamide method gives reactive group extracts from all of them.

There are indications of the presence of type or subgroup specific polysaccharides, and an extension of the method to many strains from all groups would quickly show the existence of such subdivisions where they are characterized by specific polysaccharides.

The impression gained during this work was that the antigen does not exist free in the bacterium, but is held in combination with other substances. Thus, an active extract was obtained with formamide only after heating the mixture sufficiently to break up the complex bacterial proteins. Phenol also dissolved bacteria, but here again extensive heating was necessary before water-soluble substances were extracted.

All the group antigens had roughly the same properties, but varied slightly in their ease of precipitation with acetone. None dialysed through cellophane or were destroyed by trypsin.

SUMMARY.

The formamide method for preparing group-specific extracts from hæmolytic streptococci is described. It has the advantages over existing methods of completely dissolving the bacteria, thereby giving potent extracts, and of destroying or removing protein substances which might give cross-reactions.

A preliminary purification of the group-specific polysaccharides is described.

It is suggested that the method may be applicable to all species of bacteria.

I wish to thank Dr. L. Colebrook, Director of the Laboratories, for valuable help, and all my colleagues for supplies of serum and cultures. I am also indebted to Sir P. P. Laidlaw, of the National Institute for Medical Research, for supplying large-scale cultures of group A hæmolytic streptococci.

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THE EFFECT OF SULPHONAMIDE COMPOUNDS ON CERTAIN BACTERIA *IN VITRO*.

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THE object of this work was to estimate the bactericidal and bacteriostatic effect of certain sulphonamide drugs on a number of common organisms *in vitro*. The following compounds were used: p-aminobenzenesulphonamide (sulphanilamide), p-benzylaminobenzenesulphonamide (proseptasine), disodium-p (γ-phenyl-propylamino) benzenesulphonamide-α-γ-disulphonate (soluseptasine), 4 : 4′-diaminobenzenesulphonamide (T 607) and 4 : 3′-diaminobenzenesulphonamide (T 626).

METHODS.

Saturated solutions were made by adding excess of the compound in question to broth. In the case of soluseptasine, the substance was weighed out and a 5 p.c. solution in broth made. The solutions were sterilized by filtration through a Seitz filter or by steaming for 1 hour. Because there appeared to be no difference in the results whichever method of sterilization was used, in the later experiments steaming only was used.

Six serial twofold dilutions of the solutions were made with broth for each test. 1 c.c. of mixture was used in each tube, and to this was added 0·04 c.c. of a 12-hour culture in broth of the organism to be examined. In the case of streptococci, pneumococci and the diphtheria bacillus, 5 p.c. sterile horse, serum was added to the broth used throughout.

After 24 hours' incubation at 37° C. the bacteriostatic effect was examined by comparing the tubes with a control broth tube which had also been inoculated. Subcultures were then made on to serum agar and the bactericidal effect read after a further 24 hours' incubation. The results are given in Tables I and II. A + sign means that growth was observed.

The following were the concentrations of the drugs used in the initial tubes of each test set: sulphanilamide 1 in 500; proseptasine approximately 1 in 50,000; soluseptasine 1 in 20; T 607 1 in 4400; T 626 1 in 10,000.

RESULTS.

1. No definite bactericidal or bacteriostatic effect by the above compounds was observed on the following organisms (Table I):

TABLE I.

Organism.	Number of strains examined.
B. typhosus	1
B. paratyphosus A	1
B. paratyphosus B	1
B. enteritidis	1
**B. coli communis*	6
**B. proteus vulgaris*	5
B. œrtrycke	1
B. dysenteriæ Flexner	1
B. dysenteriæ Shiga	1
B. dysenteriæ Sonne	1
Brucella abortus	1
**B. friedländer*	2
Cl. welchii	1
**Staphylococcus aureus*	3
**Streptococcus hæmolyticus*	5
**Streptococcus fæcalis*	3
**B. diphtheriæ*	2
**Micrococcus catarrhalis*	3

Cultures marked with an asterisk were freshly isolated. The other cultures were from stock cultures.

2. *Streptococcus viridans*.—Three freshly isolated strains from the throat were examined. There was no effect in two strains; in the third strain sulphanilamide was bacteriostatic in a concentration of 1 in 1000 and bactericidal in 1 in 500; proseptasine had no effect; soluseptasine was bacteriostatic in 1·25 p.c. and bactericidal in 2·5 p.c.; T 607 was bacteriostatic in 1 in 8800 and bactericidal in 1 in 4400; while T 626 was bacteriostatic in 1 in 20,000 and bactericidal in 1 in 10,000.

3. *Pneumococcus*.—Freshly isolated strains were used except for one stock culture which was being regularly passaged in mice and was highly virulent. Eight strains of pneumococci were examined and the results are given in Table II.

COMMENT.

These results show no correlation between the relative effects of these different compounds *in vitro* and those found *in vivo* by Whitby (1937). The variability of the reaction of different strains to these compounds is quite remarkable.

SUMMARY.

The effects of 5 sulphonamide compounds on 50 strains of 20 different organisms by a simple in vitro method are described. Of these 20, only in the cases of *Streptococcus viridans* and Pneumococcus Types 1 and 2 and Group 4 were any effects observed.

TABLE II.—*Pneumococcus Types.*

	Concentration.	Type 1.		Type 1.		Type 1.		Type 1.		Type 1.		Type 2.		Group 4.		Group 4.	
		Static.	Cidal.	Static.	Cidal.	Static.	Cidal.	Static.	Cidal.	Static.	Cidal.	Static.	Cidal.	Static.	Cidal.	Static.	Cidal.
Sulphanilamide	1/500	0	+	+	+	0	0	0	0	0	0	0	+	0	0	0	+
	1/1000	+	+	+	+	0	0	0	0	0	+	0	+	0	+	0	+
	1/2000	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+
	1/4000	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+
	1/8000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Proseptasine	1/50,000	+	+	+	+	0	0	+	+	+	+	+	+	0	+	+	+
	1/100,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1/200,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Soluseptasine	1/20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/40	+	+	0	+	0	0	0	+	0	0	0	+	0	0	0	+
	1/80	+	+	+	+	0	0	+	+	+	+	+	+	0	+	+	+
	1/160	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+
	1/320	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T 607.	1/4400	+	+	+	+	0	0	0	+	0	+	+	+	0	+	0	+
	1/8800	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+
	1/17,600	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+
	1/35,200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T 626.	1/10,000	+	+	+	+	0	0	+	+	+	+	+	+	0	+	0	+
	1/20,000	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+
	1/40,000	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+
	1/80,000	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+
	1/160,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

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REFERENCE.

WHITBY, L. E. H.—(1937) *Lancet*, **1**, 1517 ; and personal communication.

THE PREPARATION OF PROTECTIVE SERA AGAINST HÆMOLYTIC STREPTOCOCCI.

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THE present state of our knowledge would not justify the claim that the problem of protective sera against hæmolytic streptococci had been solved. It is true that a large number of authors have reported the production of sera possessing high titres of protective antibody, but it would appear that most of these results were obtained with strains which occupy a rather special position among the streptococci. This was certainly the case in the investigations of Yoshioka (1923) and Killian (1924), as well as in some of the author's earlier work (Loewenthal, 1932*b*), and there can be little doubt that results obtained with such strains were due to their possession of capsules. These streptococci, which behave in some respects like pneumococci, are as good antigens as the similarly encapsulated pneumococci (Etinger-Tulczynska, 1933 ; Loewenthal, 1934 ; Griffith, 1935*b*). Such strains, the prototype of which is the well-known Aronson strain, had been studied in laboratories and used in serum institutes for some thirty years before Lancefield (1933) placed them in her Group B. It has to be admitted, of course, that these particular Group B strains occurred in human infections (Loewenthal, 1932*b*, 1934 ; Griffith, 1935*b*), and have not been encountered in bovine material, but in spite of this somewhat curious position they cannot be considered as in any way representative of the streptococci producing invasive infections in man, and the results obtained with them have no real bearing on the successful serum treatment of such infections.

The attempts to produce protective sera against Group A streptococci, which group comprises the majority of strains pathogenic for man, have had

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but a limited success. Protracted courses of immunization may result in sera which protect mice against 100,000 M.L.D. when given before infection (Dochez, Avery and Lancefield, 1919; Loewenthal, 1932*b*, 1934; Lancefield, 1934), and similar results are obtained with Group C streptococci, occasionally isolated from severe human infections, but it is not possible to reproduce these results either with certainty or with all virulent strains.

The change in colony form peculiar to streptococci offered a possible explanation of this difficulty. Of the four colony forms which a hæmolytic streptococcus may show (Loewenthal, 1932*b*, 1934), only two need be considered in this paper—the mucoid (M) and the non-mucoid (N) forms. Mucoid forms of bacteria are generally bad antigens, as Friedländer's bacillus and pneumococcus Type III exemplify. With mucoid streptococci the case would appear to be much worse, for repeated attempts to produce protective sera against different strains of mucoid streptococci have invariably ended in failure (Loewenthal, 1932*b*). Since many invasive strains are isolated in the M form, and since immune sera prepared against the N form confer no protection against the mucoid variant, it is evident that the state of serum therapy must remain unsatisfactory until sera with protective power against the mucoid form are available.

The finding of capsules in young cultures of invasive Group A streptococci by Seastone (1934), confirmed and extended by Ward and Lyons (1935), was followed up in this laboratory by Pradhan (1937). Working with a highly invasive Group C strain Pradhan found that the "spreading factor", the importance of which in the mechanism of invasion has been emphasized by Duran-Reynals (1933), was present in young capsulated cultures only, and he produced evidence that the capsular substance was the factor responsible for invasiveness.

This suggested that the capsular antigen should be capable of evoking the production of protective antibody, and though the first experiments did not bear out this hypothesis, it seemed so well founded that this line of investigation has been persisted with, and the results obtained form the subject of this paper.

TECHNIQUE.

Strains of streptococci used.

The majority of the strains of hæmolytic streptococci used in this investigation were isolated from cases of severe infections treated in the wards of the London Hospital. Out of these about 30 were selected because of their high virulence for mice; it is well recognized that the virulence of these streptococci for man and the mouse does not run parallel. While confirming this, it was found possible to raise the mouse virulence of quite a large number of strains—(more than one might have been led to expect from the available data in the literature)—and it is felt that choice of medium, as well as the conditions of cultivation, influence markedly the outcome of such attempts at adaptation. For some strains I am indebted to Dr. L. Colebrook, who sent me blood from patients suffering from puerperal septicæmia. All the strains mentioned below were cultivated from the blood. In addition to these freshly isolated strains a number of old

passage-strains of Group A and also different colony-forms of a mouse streptococcus of Group C were studied, since it was necessary to compare the results described here with earlier findings.

Culture media.

Plates of 5 per cent. horse blood-agar served for the isolation and identification of these strains. Subsequently they were grown in either a meat-infusion broth containing 10 per cent. serum, or in the medium used by Ward and Lyons (1935), consisting of 2·5 per cent. neopeptone (Difco) solution containing 0·05 per cent. glucose and 5–10 per cent. serum. When the fluid media were used for the production of cultures only a few hours old, a higher percentage of serum—20–30 per cent.—was found necessary; for this purpose the neopeptone medium was definitely superior. For obvious reasons rabbit serum was used in media employed in the preparation of vaccines for the immunization of rabbits. A tryptic broth was used where ample growth was required in the absence of serum or glucose, *e. g.* in the preparation of the bacterial suspension for slide-agglutination.

Cultivation.

For the production of young cultures the inoculum should be a culture in the active phase of growth, and the medium about to be inoculated should be warmed to 37° C. When the size of the inoculum amounts to 5–10 per cent. of the medium, the capsules of the young cultures will be at their best after 2–3 hours' incubation. With a smaller inoculum, *e. g.* heart-blood from an infected mouse, a longer incubation is necessary. Since, however, the optimal time varies somewhat with each strain, the only safe rule is to watch the development of capsules by repeated microscopic examination.

Demonstration of capsules.

For stained preparations Muir's method has been relied on principally and Pradhan's photomicrographs (1937) are proof of its efficacy; it works equally well with cocci of Groups A, B or C.

A quicker and simpler method of demonstrating the capsules is the wet-film method, using 15 per cent. colloidal silver solution. For the repeated examination of cultures for the presence of capsulated cocci such a method is indispensable.

Preparation of vaccines.

(*a*) *From young cultures.*—Cultures showing an adequate growth of capsulated cocci were centrifuged in an angle centrifuge (system Lundgren); the usual laboratory centrifuges do not throw down the large capsulated cocci of young cultures from this medium of high specific gravity. The supernatant fluid was then carefully pipetted off, the sediment re-suspended in saline, and the bacteria killed by heat or by the addition of an antiseptic. When heat was employed the bacterial suspension was put into large glass ampoules,

which, after sealing, were completely immersed in a water-bath at a temperature of 55°–56° C. As thicker suspensions of streptococci are not regularly killed when exposed to this temperature for a short time the bacterial suspensions were not concentrated, the culture fluid being replaced by the same amount of saline. The length of heating finally adopted was 12 minutes; this was found to be the minimal time which could be used with safety. The vaccines were tested for sterility by incubating a large inoculum in serum broth for 2–3 days. Kept in the ice-box, they were employed for several weeks. Of the vaccines prepared by the use of chemicals it is only necessary to mention those in which formalin and merthiolate were used. Formalin was added in a concentration of 0·15 per cent., and merthiolate in one of 0·01 per cent. In both cases after 1 week in the ice-box the vaccines were sterile and ready for use. Washing the chemically-killed suspensions was considered unnecessary owing to the low concentration of the antiseptics present; further, this procedure was thought inadvisable, as it might lead to a loss of surface antigenic material.

(*b*) *From overnight* (18-*hour*) *cultures*).—These were prepared in a similar manner.

Production of immune sera.

Rabbits were immunized by intravenous injections. Six injections given in intervals of 4–5 days provided a potent serum; occasionally a few more had to be given. The initial dose was 2 c.c., and the subsequent doses were gradually increased until the final dose administered was 5–10 c.c. The animals were bled 8 days after the last injection, and the sera, inactivated by heat, were stored in the refrigerator either without any disinfectant or with merthiolate 1 in 50,000.

Titration of protective power of serum.

Mice weighing between 18–22 g. were injected intraperitoneally with the serum the day before infection. In the majority of experiments the dose given was 0·5 c.c.; occasionally a smaller amount was used. The mice were infected intraperitoneally with serial dilutions of culture. Three mice were used for each dilution in the main test of a serum, while fewer sufficed for exploratory tests. The minimal lethal dose was defined as the smallest dose which killed at least two out of three mice within a week. Only strains of high virulence were used and the results were highly consistent.

Grouping of strains.

All strains were grouped according to Lancefield's system. At first the original method was used, the antigens being prepared by extraction of the cocci by means of hot hydrochloric acid. The author is indebted to Mrs. Lancefield for a supply of group sera which were used in parallel with sera prepared by the author. Strains not falling into Group A (a few B and a certain number of C strains) were eliminated.

Arising out of observations made in another connection (Loewenthal,

1937), a slightly different technique for the preparation of the antigen was later adopted. The cocci, obtained by centrifuging 100–200 c.c. of an overnight culture, were resuspended in a few c.c. of saline and centrifuged again immediately. The supernatant was then pipetted off and the sediment discarded. Three volumes of alcohol were added and, after precipitation was complete, the precipitate was removed by centrifuging and a further 20 volumes of alcohol were added. Next day the resulting precipitate was spun down in the centrifuge and the sediment, freed as completely as possible from liquid, was dissolved in a small quantity of distilled water. This preparation contains sufficient C-substance for the performance of a precipitin test.

Typing of strains.

The strains were first of all typed by means of slide agglutination, using sera prepared by the author. The cultures were grown in plain broth, tryptic broth, or serum broth, according to their tendency to form granular suspensions, and the cocci were allowed to settle spontaneously, or were centrifuged at low speed to avoid packing. A loopful of undiluted serum, prepared by immunization with young cultures, was mixed on a slide with a drop of the bacterial suspension and the reaction observed without using the microscope. After the strains had been typed in this manner they were sent to Dr. F. Griffith, who kindly undertook to type them with his sera. The designation of types used in this paper follows Griffith's classification.

EXPERIMENTAL.

Since frequent references will be made to young cultures in contradistinction to ordinary or overnight cultures, it might be advisable to summarize here their main differences.

In every medium tested the young culture shows the more homogeneous growth ; even strains marked by very granular growth when cultivated for 18 hours, will be found growing with uniform turbidity after a few hours of incubation. Microscopically, diplococci and short undistorted chains predominate in young cultures, while long chains and agglutinated clumps are found in ordinary cultures. Undoubtedly the most striking difference is the presence in young cultures of large capsules, the size of which is best recognized by means of the wet-film method. The size of a young coccus together with its capsule is at least twice that of a coccus grown overnight. This seems to account for the fact that a suspension of young cocci is not much less opaque than one made up from an overnight culture, though the number of cocci is much smaller in the younger culture. The size of the sediment obtained from a young culture is much bigger than that from an equal amount of overnight culture. The cause of this, at first sight, paradoxical phenomenon is that the young cocci with their complement of viscous capsular material cannot be so closely packed in the centrifuge as the " naked " cocci of an ordinary culture. While the deposit of an old culture still sticks to the bottom of the tube when it is inverted, the sediment from a young culture will flow down the walls of the tube.

Comparison of the Efficacy of Vaccines made from Young and Old Cultures in the Production of Protective Sera.

Experiments with mucoid strains.

The strain of streptococcus used in these experiments (L. 193) was isolated from a case of puerperal septicæmia in 1935. When first isolated it was in the mucoid phrase, and the large watery colonies on blood-agar and the diffuse growth in fluid media, characteristic of this phase, have remained a feature of this strain. It is strongly hæmolytic, is of high virulence for mice and belongs to Griffith's Type 13. It was selected for the purposes of these experiments, because previous attempts to produce a protective antiserum against it had proved unavailing ; it was already mentioned that it is impossible by the usual methods to produce a potent serum against mucoid streptococci. Suspensions prepared from young capsulated cultures and overnight cultures were killed by exposing them to a temperature of 55° C. for varying periods of time, or by the addition of formalin (0·15 per cent.) or merthiolate (0·01 per cent.). Rabbits were then injected intravenously with these preparations and bled 8 days after the last injection. It seems hardly necessary to give in detail the results of the tests of all the sera prepared, since most of them were devoid of protective antibody. Instead a *résumé* of the findings is given in Table I, from which it will be seen that, of the various vaccines, only the one

TABLE I.—*Protective Power of the Sera of Rabbits Immunized with various Vaccines Prepared from Strain L. 193.*

Preparation of vaccine.		Protective antibodies in serum.
Age of culture.	How killed.	
Overnight	Heat : 45 mins., 55° C.	None
,,	Heat : 12 mins., 55° C.	,,
,,	Formalin 0·15 per cent.	,,
,,	Merthiolate 0·01 per cent.	,,
Young	Heat : 45 mins., 55° C.	,,
,,	Heat : 12 mins., 55° C.	Protection against 100,000 M.L.D.
,,	Formalin 0·15 per cent.	None
,,	Merthiolate 0·01 per cent.	Irregular protection against 1000 M.L.D.

prepared from young cultures and killed by heating for 12 minutes at 55° C. produced protective sera of value. The failure with vaccines prepared from overnight cultures is in conformity with previous experience, but the lack of success encountered with the suspensions of young cultures killed by 45 minutes' heating at 55° C. or by the addition of formalin in low concentration was unexpected. These young cultures were composed of fully capsulated cocci, and it was not thought that this treatment would destroy the capsular antigen. However, the belief in the importance of this antigen was unshaken by these earlier findings, and it was ultimately confirmed by the demonstration

that heating the capsulated organisms just sufficiently long to kill them, *i. e.* 12 minutes at 55° C., provided a potent antigen. The details recorded in Table II of the titration in mice of the protective power of three sera prepared against this mucoid streptococcus L. 193 serve to illustrate this.

TABLE II.—*Comparison of Protective Value of Sera of Rabbits Immunized with Old and Young Cultures of Strain L. 193 killed by Heat.*

Dose of culture of L. 193 in c.c. injected 18 hours after serum.	Mice receiving 0·5 c.c. serum of—												
	Normal rabbit.			Rabbit 98. Immunized with overnight culture heated for 12 mins. at 55° C.			Rabbit 102. Immunized with young culture heated for 45 mins. at 55° C.			Rabbit 174. Immunized with young culture heated for 12 mins. at 55° C.		
10^{-2}	..	..	..	D1	D1	D1	D1	D1	D1	D1	D1	D2
10^{-3}	..	..	..	D1	D1	D1	D1	D1	D1	S	S	S
10^{-4}	..	.	..	D1	D2	D2	D2	D2	D2	S	S	S
10^{-5}	D1	D2	D2	D1	D2	D2	D1	D2	D2	D2	S	S
10^{-6}	D2	D2	D2	D2	D2	D2	D2	D2	D3	S	S	S
10^{-7}	D2	D2	D2	D2	D2	D2	D2	D2	D2	S	S	S
10^{-8}	D2	D3	D3	D2	D2	D2	D2	D2	D2	S	S	S
10^{-9}	D3	S	S	D2	D2	S	S	S	S	S	S	S

.. = Not tested. S = Survived. D = Died. Numerals indicate the number of days before the death of the animal after infection. The same symbols are used in the following tables.

It will be seen that the serum of the rabbit 102, immunized with young cultures heated at 55° C. for 45 minutes, had no more protective power than normal rabbit serum, whereas rabbit 174 immunized with the same culture, but heated for only 12 minutes, provided a serum protecting mice against 100,000 M.L.D. of the homologous streptococcus. Since the serum of rabbit 98, which was injected with overnight culture heated for only 12 minutes at 55° C., was devoid of protective antibody, and since the difference between young and old cultures is the capsulation of the cocci in the former, it seems clear that the antigen responsible for the production of the protective antibody is associated with the intact capsule, and that the activity of this antigen is readily destroyed by heat.

It should be emphasized that this is not an isolated example. A considerable number of strains of Group A hæmolytic streptococci, against which a protective serum could not be made in the rabbit by the usual method, have been examined in the same way as L. 193 with a similar result. And that this important finding is not confined to Group A strains is shown by the result of an experiment made with a Group C strain recorded in Table III.

The strain used in these experiments was isolated from a mouse epidemic seven years ago. Repeated attempts to produce a protective serum against the mucoid variant of this strain had, in the past, ended in complete failure (Loewenthal, 1932*a* and *b* ; Pradhan, unpublished). It is true that Pradhan attempted to immunize rabbits with young cultures of this strain, but he failed to obtain a protective serum, since the detrimental effect on the vaccine of prolonged heating was not then appreciated. However, the data given in Table III leave no doubt that, as in the case of Group A strains, a highly

TABLE III.—*Protective Value of Sera of Rabbits Immunized with Vaccines prepared from Young and Old Cultures of the Mucoid Variant of a Group C Streptococcus.*

Dose of culture in c.c. injected 18 hours after serum.	Mice receiving 0·5 c.c. serum of—									
	Normal rabbit.			Rabbit 54. Immunized with overnight culture heated for 12 min. at 55° C.			Rabbit 226. Immunized with young culture heated for 12 mins. at 55° C.			
10^{-2}	..	..	..	..	..	..	D2	D2	D2	
10^{-3}	..	..	..	..	..	..	D2	D2	D3	
10^{-4}	..	..	..	..	..	..	S	S	S	
10^{-5}	D2	D2	D2	D2	D2	D2	S	S	S	
10^{-6}	D2	D2	D2	D2	D2	D2	D3	S	S	
10^{-7}	D2	D3	D3	D2	D2	D2	S	S	S	
10^{-8}	D2	D3	D3	D2	D2	D2	S	S	S	
10^{-9}	D3	D3	S	D2	D3	D3	S	S	S	

potent serum can be prepared in the rabbit against the mucoid variant of this mouse streptococcus provided the suspension used for immunization is prepared from young capsulated cocci killed by exposure to a temperature of 55° C. for 12 minutes.

Experiments with non-mucoid strains.

It seemed clear that the difficulty of producing effective sera in the rabbit against the mucoid variants of hæmolytic streptococci could be overcome by the use of cautiously heat-killed suspensions of young cultures. The question now arose as to what results this new method of immunization would give with non-mucoid hæmolytic streptococci against which protective sera had been obtained by the older methods. Using young cultures of such strains cautiously killed by heat, would it not be possible to produce high-titre protective sera by a shorter course of immunization than had been found necessary with the vaccines made from older cultures? To obtain an answer to this question experiments were carried out with three strains. One of these, B. 8, was a laboratory passage strain; it belonged to Lancefield's Group A and Griffith's Type 25. The second, B. 9, also a Group A strain, was referred to in a previous publication (Loewenthal, 1934) as streptococcus "Krueger", whilst the third was the non-mucoid (N) form of the Group C mouse streptococcus (Loewenthal, 1932*a* and *b*), the mucoid variant of which was used in the experiment described above. In the case of all three strains it was found possible to produce highly potent protective sera in the rabbit by six injections of cautiously killed encapsulated young cocci, whereas the same number of injections of a vaccine similarly prepared from overnight cultures yielded sera of low protective value; only a prolonged course of immunization with the latter preparation provided highly potent sera. The results obtained with the strain B. 8, given in Table IV, serve to illustrate this superiority of the young culture vaccines of these non-mucoid strains.

TABLE IV.—*Comparison of Protective Power of Sera from Rabbits Immunized with cautiously Heat-killed Suspensions of Young and Overnight Cultures of a Non-mucoid Streptococcus (B. 8).*

Dose of culture in c.c. injected 18 hours after serum.	Mice receiving 0·5 c.c. serum of—											
	Normal rabbit.			Rabbit 254. Given 6 injections of young culture heated for 12 mins. at 55° C.			Rabbit 259. Given 6 injections of overnight culture heated for 12 mins. at 55° C.			Rabbit 770. Given 18 injections of overnight culture heated for 12 mins. at 55° C.		
10^{-2}	..	..	..	D2	D2	D2	D2	D2	D2	D2	D2	D2
10^{-3}	..	..	..	S	S	S	D2	D2	D2	D2	S	S
10^{-4}	..	..	..	S	S	S	D2	D2	D2	S	S	S
10^{-5}	D2	D2	D2	D3	S	S	D2	S	S	D3	S	S
10^{-6}	D2	D2	D2	S	S	S	D2	D2	S	S	S	S
10^{-7}	D2	D2	D2	S	S	S	D3	S	S	S	S	S
10^{-8}	D2	D3	D3	S	S	S	S	S	S	S	S	S
10^{-9}	S	S	S	..	..	..	..	..	..	..	..	..

The Specificity of the Protective Sera made with Young Cultures.

There is already evidence from the literature that those protective sera which it has been possible to prepare against hæmolytic streptococci are type-specific in their action. It was necessary, however, to find out whether the protective sera made in rabbits with mucoid streptococci by the new method were also type-specific. The possibility existed that they might show some overlapping in protective action, though this possibility was thought to be remote. Three strains of mucoid streptococci were used in this experiment. L. 193 has already been described in an earlier experiment; the other two—L. 212 and L. 371—although untypeable by Griffith's sera, were shown to be of different types by means of sera prepared by the author; L. 193, as will be remembered, belonged to Griffith's Type 13. L. 212 was isolated from a case of post-operative septicæmia, L. 371 from a case of puerperal septicæmia, and both were highly virulent for mice and were typical mucoid strains; against neither had it been possible to prepare protective sera by the old methods. Rabbits were injected with cautiously heat-killed suspensions made from young cultures of these three strains. The result of the titration in mice of the protective power of these three sera, given in Table V, leaves no doubt that the sera are strictly type-specific in their action.

The Effect of Simultaneous Immunization with Three Strains.

It is obvious that the multiplicity of types among hæmolytic streptococci responsible for human infections and the type-specificity of protective sera would greatly handicap the elaboration of a practical serum therapy. Successful simultaneous immunization with a number of strains would go far to overcome this difficulty. With a view to exploring this possibility, a rabbit was immunized by the new method with the three mucoid strains L. 193, L. 212 and L. 371 simultaneously. As will be seen from the data given in Table VI the serum of this rabbit acquired a high degree of protective power against all three strains.

TABLE V.—*The Type-specificity of Sera prepared with cautiously Heat-killed Suspensions of Young Cultures.*

Dose of culture in c.c. injected 18 hours after serum.	Mice infected with—																							
	Strain L. 193, having received—								Strain L. 212, having received—								Strain L. 371, having received—							
	Normal serum.		serum prepared against strains—						Normal serum.		serum prepared against strains—						Norma serum.		serum prepared against strains—					
			L. 193.		L. 212.		L. 371.				L. 193.		L. 212.		L. 371.				L. 193.		L. 212.		L. 371.	
10^{-2}	..	..	D2	D2	..	..	..	..	..	..	..	..	D2	D2	..	..	..	..	..	..	..	..	D1	D1
10^{-3}	..	..	S	S	..	..	..	..	..	..	..	..	D3	S	..	..	..	..	..	..	..	..	D2	D2
10^{-4}	..	..	S	S	D2	D2	D2	D2	..	..	D2	D2	S	S	D2	D2	..	..	D2	D2	D2	D2	S	S
10^{-5}	D2	D2	S	S	D2	D2	D2	D2	D2	D2	D2	D2	S	S	D2	D2	D2	D2	D2	D2	D2	D2	S	S
10^{-6}	D2	D2	S	S	D2	D3	D2	D2	D2	D2	D2	D3	S	S	D2	D2	D2	D2	D2	D2	D2	D2	S	S
10^{-7}	D2	D3	S	S	D2	D2	D2	D2	D2	D2	D2	D2	S	S	D3	D3	D2	D2	D3	D3	D2	D3	S	S
10^{-8}	D2	D2	S	S	D2	D2	D2	D2	D3	D2	D2	D2	S	S	D2	D3	D2	D2	D2	D3	D2	D3	S	S
10^{-9}	S	S	..	..	..	..	..	..	D2	S	..	..	..	..	..	..	S	S	..	..	..	..	..	..

TABLE VI.—*Protective Value of Serum of Rabbit 256 Immunized Simultaneously against Strains L. 193, L. 212 and L. 371.*

Dose of culture in c.c. injected 18 hours after serum.	Mice infected with—											
	Strain L. 193, having received 0·5 c.c.—				Strain L. 212, having received 0·5 c.c.—				Strain L. 371, having received 0·5 c.c.—			
	Normal serum.		Antiserum 256.		Normal serum.		Antiserum 256.		Normal serum.		Antiserum 256.	
10^{-2}	..	..	D1	D1	..	..	D1	D1	..	..	D2	D2
10^{-3}	..	..	D2	S	..	..	D2	S	..	..	S	S
10^{-4}	..	..	S	S	..	..	S	S	..	..	S	S
10^{-5}	D2	D2	S	S	D2	D2	S	S	D2	D2	S	S
10^{-6}	D2	D2	S	S	D2	D3	S	S	D3	D3	S	S
10^{-7}	D2	D2	S	S	D2	D2	S	S	D2	D3	S	S
10^{-8}	D2	D3	S	S	D2	D2	S	S	D3	D3	S	S
10^{-9}	S	S	..	..	D2	D3	..	..	S	S	..	..

Precipitins and Agglutinins in the Sera prepared by the New Method.

Although this investigation was concerned principally with the protective antibody in the sera, observations were also made on their precipitin and agglutinin content which will be briefly referred to.

Precipitins.

After about six injections, sera prepared with young cultures possess sufficient antibody to the C-substance for the performance of the precipitin test. Though the reaction is weaker than that given by sera prepared by the usual method, it appears to be more specific, since no cross-reactions could be observed.

Agglutinins.

There is evidence that the protective power and agglutinin content of anti-streptococcal sera do not run parallel; a serum with a high agglutinin titre may be devoid of protective antibody. It has been the practice to use overnight cultures in agglutination tests in this investigation, as it has been found that young cultures are quite unsuited to this purpose. As Pradhan (1937) observed, young cultures of encapsulated cocci are not agglutinated by the sera made with overnight cultures, and to this the author can now add that the same seems to be true of sera made by the new method with young cultures.

But for the purpose of typing by slide-agglutination the new sera proved satisfactory. Rabbits, which had received only a few injections of young cocci, provided sera which agglutinated suspensions prepared from overnight cultures in a type-specific manner. After about four injections the sera had acquired agglutinins, and the reactions they gave were sufficiently clear-cut

as to render unnecessary the preliminary absorption with heterologous types which Griffith employs in the preparation of his typing sera. With further injections the sera gradually lost their type-specificity and cross-reactions appeared. Another point in favour of the new sera for the purpose of slide-agglutination is that, whereas by the old method it is not possible to prepare typing sera against every strain, no difficulties in this respect were found with sera made with young cultures. On the other hand there is a certain disadvantage in the low agglutinin titre of sera so prepared, and the best results would probably be obtained by augmenting the number of injections of young-culture vaccine and absorbing the sera with C-substance, as recommended by Pauli and Coburn (1937).

DISCUSSION.

The experiments described have given such clear-cut and consistent findings that any discussion of them is hardly necessary. They seem ample proof of the correctness of the assumption, made at the begining of this investigation, that young capsulated cocci are more effective in producing protective sera than those from overnight cultures. Not only were protective antibodies formed more quickly and more regularly in the case of non-mucoid strains, but it was possible for the first time to produce protective sera against streptococci in the mucoid phase.

The fact that the antigen eliciting the formation of the protective antibody, which is probably the unaltered type-specific antigen, is heat-labile is really not surprising, since it would appear to be a protein in the case of Group A and C streptococci (Lancefield, 1928, 1933; Stamp and Hendry, 1937). Mudd and his fellow workers (1937) have also observed that the factor responsible for removing the phagocytosis-promoting antibody from immune serum (probably the type-specific antigen) is very heat-labile.

The precautions taken to preserve unaltered the type-specific antigen led to the finding of a second labile antigenic substance. This was isolated from mucoid streptococci and found to be of polysaccharide nature. It is identical with the polysaccharide described by Kendall, Heidelberger and Dawson (1937), as was shown by serological tests with their preparation, which they most generously placed at the disposal of the author. The fact that it was found to be serologically inactive by these workers is probably due to its extreme lability in the natural state. Not only is it not type-specific, but it occurs in the mucoid substance produced by other bacteria such as pneumococcus Type III. It does not appear to participate in the production of the protective antibody, since complete removal of the precipitins in a serum by absorption with the polysaccharide does not lower its protective power. It is proposed to give a more detailed account of this interesting substance in a subsequent publication.

Future work will have to aim at a further improvement in the potency of sera, and at the elaboration of simultaneous immunization to overcome the difficulty presented by the multiplicity of types.

CONCLUSIONS.

1. The antigen responsible for the production of the antibody effective against the invasiveness of hæmolytic streptococci is intimately associated with the capsule which these cocci develop in the early hours of growth in culture.

2. This antigen is extremely labile, being readily destroyed by heat and low concentrations of formalin or merthiolate.

3. Exposure of a suspension of young encapsulated cocci to a temperature of 55° C. for 12 minutes kills the organisms but leaves the capsular antigen intact. More prolonged heating at this temperature rapidly destroys this antigen.

4. With suspensions of cautiously heat-killed young cultures it has been possible for the first time to prepare potent protective sera against hæmolytic streptococci in the mucoid phase. This method of immunization also gives rise to protective antibody against non-mucoid strains more rapidly and regularly than the old methods.

5. The result of a single experiment suggests that the production of a potent polyvalent serum by simultaneous immunization with a number of strains is possible.

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SPECIFIC COMPLEMENT-FIXATION WITH SHOPE'S FIBROMA VIRUS AND ITS RELATIONSHIP TO VIRUS-NEUTRALIZING PROPERTIES OF IMMUNE SERA.

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THE existence of specific complement-fixing antibodies has already been demonstrated in the sera of human subjects and in animals following recovery from certain virus infections.

The present paper records the development of specific complement-fixing antibodies in the sera of rabbits after immunization with Shope's fibroma virus.

METHODS.

Strain of virus.—Two varieties of this virus have been used in the experiments, namely, the inflammatory (I A) strain and the fibromatosis (O A) type.

Method of immunization.—1 gm. of desiccated rabbits' testis infected with the I A strain of virus active to an end-titre of 1 : 1,000,000 dilution in broth was used as antigen. This was ground in a mortar, emulsified in 9 c.c. of 0·86 per cent. saline, centrifuged at 3000 r.p.m. for 30 min., the supernatant fluid removed, and used for immunization as live virus. A similar 1 : 10 dilution of infected tissue was prepared, heated to 55° C. for 60 min., tested for non-pathogenicity and used for immunization. Rabbits inoculated subcutaneously with this extract failed to show any cutaneous reactions, and the virus was therefore presumed to be inactive or dead.

The antigen control consisted of a 1 : 10 dilution of normal desiccated rabbits' testicle which was used for injecting control animals.

Twelve rabbits were immunized with live virus, a similar number with inactive (heated) virus and 6 with normal rabbits' testicle tissue. Animals were injected at weekly intervals, and each received 0·5 c.c. subcutaneously, 1 c.c. intravenously, 2 and 4 c.c. intraperitoneally respectively.

Sampling of blood.—Sample bleedings were made at 2, 3 and 4 weeks after the date of the first injection, and a final specimen was taken 8 weeks after the last inoculation of virus. The serum was heated at 55° C. for 30 min. and about 1 c.c. used for an immediate complement-fixation test, the remainder being stored in the refrigerator.

Technique of complement-fixation tests.—A fixed volume of serum and antigen and varying amounts of complement have been employed in the tests. The degree of the reaction has thus been assessed in terms of the number of m.h.d. of complement fixed.

Reagents.

Serum.—0·05 c.c. of rabbits' serum previously heated to 55° C. for 30 min. obtained as described above was used.

Antigen.—A 1 : 1000 dilution of desiccated rabbits' testicular tissue was made up in 0·86 per cent. sodium chloride solution, centrifuged at 3000 r.p.m. for 30 min. and the supernatant fluid removed. The opalescence shown by different specimens of testis tended to vary, and it was arbitrarily decided to standardize the suspension to the density of Brown's opacity tube No. 4. In practice this was found to be the maximum degree of opalescence permissible without interfering with the reading of hæmolytic effects on the mixture.

Complement.—Guinea-pigs' complement which had been preserved after the method described by Sonnenschein (1930) was used throughout. This was titrated on each occasion before use, and the minimum hæmolytic dose (m.h.d.) usually varied from 0·005 to 0·006 c.c.

Hæmolytic system.—A 3 p.c. saline suspension of thrice washed sheep erythrocytes was sensitized with a standard amboceptor (B. W. & Co.), 0·35 c.c. being used to sensitize 50 c.c. of the suspension.

Procedure.—To 4 tubes containing 0·05 c.c. of serum were added 4, 6, 8 and 10 m.h.d. of complement respectively, followed by 0·5 c.c. of antigen to each tube. The effect of adding antigen last was also tried in a number of tests, and found to make no difference.

Control.—The serum control consisted of 2 tubes containing 4 and 6 m.h.d. of complement plus 0·5 c.c. of saline. The antigen controls comprised 2 tubes with 0·5 c.c. of antigen and 4 and 6 m.h.d. of complement respectively.

Length of time for which mixtures were incubated.—The above mixtures were incubated at 37° C. for 1½ hours, and then 0·5 c.c. of sensitized cells was added to each. The tubes were next re-incubated at 37° C. for 1½ hours, removed from the incubator and the result read. A final reading was made the next day after the tubes had been standing for 24 hours at room temperature.

Interpretation of results.—Although certain sera with antigen fixed as much as 8 m.h.d. of complement, the reaction as a whole was a weak one. This was probably due to the fact that only dilute semi-opalescent extracts of Shope testis could be used as antigen, more concentrated tissue suspensions masking hæmolysis. Fixation of less than 4 m.h.d. complement was ignored since normal rabbit serum sometimes fixed 2 or 3 m.h.d.

RESULTS.

Table I gives the results obtained with sera of rabbits which had been repeatedly immunized with living I A Shope virus.

The serum of each animal was tested for complement-fixing antibodies before they had been immunized, and 2, 3 and 4 weeks after the date of the

first injection (see columns 2, 3, 4). The results of the tests 8 weeks after the rabbits received the last dose of virus (or 12 weeks after the first dose) are also recorded.

Tests with two control sera were incorporated in each series; one serum was obtained from a healthy uninoculated rabbit, and the other from a rabbit which had received four repeated injections of normal rabbits' testicular extract.

TABLE I.

Rabbit B.

MHD complement.	Before immunization.	Weeks after first injection. 2.	3.	4.	After interval of 8 weeks. 12.
4	−	+	++	++	−
6	−	−	−	−	−
8	−	−	−	−	−
10	−	−	−	−	−
Serum control 4	−	−	−	−	−
Serum control 6	−	−	−	−	−
Antigen control 4	−	−	−	−	−
Antigen control 6	−	−	−	−	−

Rabbit E.

MHD complement.	Before immunization.	Weeks after first injection. 2.	3.	4.	After interval of 8 weeks. 12.
4	−	+	+++	+++	..
6	−	−	++	++	−
8	−	−	+	+	−
10	−	−	−	+	−
Serum control 4	−	−	−	−	−
Serum control 6	−	−	−	−	−
Antigen control 4	−	−	−	−	−
Antigen control 6	−	−	−	−	−

− = complete lysis. + = partial lysis. ++ = trace of lysis. +++ = no lysis.

From the above and other data it was concluded that rabbits repeatedly injected with live Shope (I A) strain of virus develop complement-fixing antibodies for that agent. These were occasionally observed as early as two weeks after the first injection of live virus had been administered (Rabbit B), but were more usually present 3 weeks after the first inoculation, and were well marked at the fourth week (Rabbit E). In Rabbit A and 3 others which are not included in the table, notwithstanding repeated doses of live virus, no complement-fixing antibodies appeared, and in animal B only 4 m.h.d. were fixed. All 4 animals showed a cutaneous reaction after the first dose of virus had been given, and failed to show any further skin response when injected with active virus after their course of immunization. Thus, although the

animals appeared to have developed active immunity to Shope virus, it is interesting to record that no complement-fixing antibodies were demonstrable in their sera. The sera of these animals were later examined for virus-neutralizing properties and reference to these is made below.

Result of Immunization with Heated (Killed) Virus.

As stated earlier, 12 rabbits were inoculated with testicular extract containing Shope virus I A strain, which had been previously heated to 55° C. for 60 min. As before, sample bleedings were made from the animals before they were inoculated, and after 2, 3, 4 and 12 weeks respectively. Sera were tested for the presence of complement-fixing antibodies as in the case of rabbits which had been immunized with the live virus. The results are tabulated below.

TABLE II.

	Rabbit H.					
MHD complement tested.	Control serum before immunization.	Weeks after first injection. 2.	3.	4.	After interval of 8 weeks. 12.	Serum control.	Antigen control.
4	—	—	+	++	—	—	—
6	—	—	+	++	—	—	—
8	—	—	—	+	—	..	..
10	—	—	—	—	—	..	..

The foregoing result, noted in rabbit H, was very similar to the response elicited in three others. These indicated that the inoculation of heated (presumably killed) virus stimulated the production of specific complement-fixing antibodies. Their occurrence, however, was slightly delayed in comparison to the response following injection of live virus, and they did not appear until the third week. Their presence was well marked at the fourth week, and two months after the last dose of heated virus they had completely disappeared. In two rabbits not mentioned in the table, no complement-fixing antibodies could be demonstrated at any period.

Cross Complement-fixation Tests with I A and O A Strains of Virus.

Tests were performed in order to discover whether the sera of rabbits which had been immunized with I A strain of virus would fix complement in the presence of antigen consisting of a saline extract of testicle infected with the O A virus.

The serum of each animal was accordingly tested against I A and O A testis antigens.

Two serum controls were also included, one derived from a normal rabbit and the other from one which had been repeatedly injected with normal rabbit testis.

TABLE III.

Serum of rabbit injected with living Shope fibroma virus strain I A with—

M.H.D. of complement.	I A. B	I A. A	O A. B	O A. A	Normal testis. B	Normal testis. A	Saline.
4	—	++	—	++	—	—	—
6	—	+	—	+	—	—	—
8	—	+	—	—	—	—	..
10	—	—	—	—	—	—	..

Serum of rabbit injected with heated Shope fibroma virus strain I A with—

M.H.D. of complement.	I A. B	I A. A	O A. B	O A. A	Normal testis. B	Normal testis. A	Saline.
4	—	++	—	++	—	—	—
6	—	+	—	+	—	—	—
8	—	+	—	—	—	—	..
10	—	—	—	—	—	—	..

M.H.D. of complement.	Serum of normal rabbit with— I A.	O A.	Normal testis.	Saline.	Antigen control. I A.	O. A.	Normal testis.
4	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—
8	—	—	—	—	..	..	..
10	—	—	—	—	..	..	..

B = Before immunization. A = after immunization 2 weeks after the last dose.

The results indicated that the sera of rabbits which have been immunized with the I A strain of virus (live or heated) show cross complement-fixation for both the I A as well as the O A virus, though to a lesser extent. It was also possible to show that rabbits inoculated with the O A strain revealed similar cross complement-fixation for I A antigen. There was no evidence that the sera of rabbits immunized against Shope virus produced non-specific complement-fixation with either normal testis or that containing vaccinia virus.

The Relationship of Complement-fixing Antibodies to Virus-neutralizing Properties of Immune Sera.

A number of rabbits which had been immunized with the I A strain of virus were bled and their sera tested for virus-neutralizing antibodies. The group included animals which had been immunized with (*a*) live virus, (*b*)

TABLE IV.—*Diameter of Erythematous Reaction Produced by Injection of Diluted Virus Material.*

Sera tested.	Skin reaction after—	1 : 20.	1 : 200.	1 : 2000.	1 : 20,000.	1 : 2,000,000.
		(cm.)	(cm.)	(cm.)	(cm.)	(cm.)
Serum of rabbit (A) before immunization with live Shope virus (I A strain) Complement fixed with Shope virus <4 MHD	5 days	2	1·5	1	—	—
	10 ,,	2	1	0·5	—	—
Serum of same rabbit (A) AFTER immunization with live Shope virus I A strain Complement fixed with Shope virus <4 MHD	5 days	—	—	—	—	—
	10 ,,	—	—	—	—	—
Serum of rabbit (B) before immunization with live Shope virus I A strain Complement fixed with Shope virus <4 MHD	5 ,,	2	1·5	1	0·5	—
	10 ,,	2·5	1·5	1	0·7	—
Serum of rabbit (B) AFTER immunization with live Shope virus I A strain Complement fixed with Shope virus —4 MHD	5 ,,	1	—	—	—	—
	10 ,,	1·5	0·5	—	—	—
Serum of rabbit (E) before immunization with heated Shope virus I A strain Complement fixed with Shope virus <4 MHD	5 ,,	2	1·5	1	0·5	—
	10 ,,	2·5	2	1·5	1	—
Serum of rabbit (E) AFTER immunization with heated Shope virus (I A strain) Complement fixed with Shope virus = 10 MHD	5 ,,	0·5	—	—	—	—
	10 ,,	0·5	—	—	—	—
Serum of rabbit (K) before immunization with live Shope virus (I A strain) Complement fixed with Shope virus <4 MHD	5 ,,	1·5	1	1·5	—	—
	10 ,,	2	0·5	1	0·5	—
Serum of rabbit (K) AFTER immunization with live Shope virus I A strain Complement fixed with Shope virus = 8MHD	5 ,,	0·5	—	—	—	—
	10 ,,	0·75	—	—	—	—

heated (killed) virus, (*c*) those which showed complement-fixing antibodies in their sera, and (*d*) those in which no complement-fixing antibodies were demonstrable, notwithstanding the repeated injections of virus they had received (*e. g.* Rabbit A). The control consisted of serum obtained before immunization.

This experiment made it possible to estimate the degree of virus neutralization by a serum and to correlate this result with the complement-fixing power.

Technique.—0·25 c.c. of serum, heated to 55° C. for 30 min., was added to 0·25 c.c. of falling decimal dilutions of virus ranging from 1 : 20 to 1 : 2,000,000. The mixtures were thoroughly shaken, and each dilution was inoculated into the shaved skin of a rabbit. Two sets of titrations were performed on each animal, and the size of the papule was measured on the fifth and tenth days after inoculation.

The results indicate that after repeated injections of either living or killed virus the serum of the animal acquires neutralizing properties for the virus, and that the activity is unrelated to the complement-fixing power. For example, Rabbit A before immunization possessed neither complement-fixing antibodies nor virus-neutralizing properties in its serum, but after immunization with live virus, although no complement-fixing antibodies were developed, the serum of this rabbit had strong neutralizing power.

Shope fibroma virus immune sera thus could be showed to possess virus-neutralizing properties without evidence of specific complement-fixing antibodies. It has not been possible, however, to obtain sera containing complement-fixing properties without also possessing virus neutralizing properties. To summarize, it may be stated that complement-fixing antibodies may or may not appear in the serum of an animal immunized with Shope fibroma virus, but when present, the serum also possesses virus-neutralizing properties. The latter property has been demonstrated following inoculation with either living or dead virus, and may occur in marked degree without the serum exhibiting any evidence of specific complement-fixation.

DISCUSSION.

The facts recorded are plain, and are in agreement with similar findings of earlier workers in respect of other virus diseases. The present work has shown that complement-fixing antibodies appear in the sera of rabbits immunized either with living or dead Shope fibroma virus. At one time it was thought that dead viruses were devoid of antigenic value. This opinion has been dispelled by the work of Bedson (1931), who clearly demonstrated the immunizing properties of killed herpes virus. He moreover showed that whereas a suspension of virus material killed by heating at 60°–100° C. lost its antigenic power, it did not do so if formalin was added to the suspension before heating it. The immunizing value of killed virus has also been demonstrated in the case of influenza by Andrewes (1937), Wilson Smith (1937) and Fairbrother and Hoyle (1937). Both complement-fixing and virus-neutralizing properties developed in the sera of rabbits after repeated doses of either live or heat-killed Shope fibroma virus. It is interesting to record that although some of the

rabbits showed virus-neutralizing antibodies in their sera, no complement-fixation could be demonstrated at the same time. The converse, however, was not encountered, for every serum possessing complement-fixing antibodies also in high dilution neutralized the virus. The former were usually demonstrable 2–3 weeks after the first injection, and generally disappeared about 8 weeks after the last dose of virus had been administered.

Bedson (1933), on the contrary, working with psittacosis virus found that although convalescent patients' sera contained complement-fixing properties, it possessed a low virus-neutralizing effect. These facts tend to suggest that the immunity mechanism prevailing in psittacosis infection might be different from that produced by Shope fibroma virus.

CONCLUSIONS.

1. Rabbits immunized with live Shope fibroma virus of either the I A or O A strain developed complement-fixing as well as virus-neutralizing properties in their sera.

2. Both the I A and O A strains were indistinguishable from their complement-fixation reactions.

3. Complement-fixing antibodies appeared about 2–3 weeks after injection. They usually disappeared about 8 weeks after the last dose of virus was given.

4. Shope fibroma virus when heated to 55° C. for one hour still retains its antigenic properties, giving rise to both complement-fixing and neutralizing antibodies.

5. Notwithstanding repeated doses of living (or dead) virus, no complement-fixing antibodies appeared in the sera of certain rabbits.

6. The sera of such animals, however, showed a powerful virus-neutralizing effect.

7. Whereas all the sera yielding complement-fixation also neutralized the virus, none were found in which complement-fixing antibodies were present without showing virus neutralization also.

It is with pleasure that I desire to thank Dr. C. H. Andrewes, who kindy supplied me with specimens of Shope fibroma and papilloma virus. Also Prof. Mackie for many helpful suggestions received. The work has been done during receipt of an expenses grant from the Medical Research Council.

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THE SEROLOGICAL PROPERTIES OF A POLYSACCHARIDE ISOLATED FROM THE MUCOID SUBSTANCE OF HÆMOLYTIC STREPTOCOCCI.

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In a previous paper (Loewenthal, 1938) reference has been made to the isolation of a serologically active material of polysaccharide nature from mucoid streptococci. The results of earlier work (Loewenthal, 1932, 1937 ; Pradhan, unpublished) had suggested to the author the possibility that the mucoid material elaborated by these hæmolytic streptococci contained an antigen which played an important part in the invasiveness of these strains. The inability to produce protective sera against streptococci in the mucoid phase, and the fact that sera which protected against a non-mucoid or N form entirely failed to do so against an infection with the mucoid variant of the same strain, gave some support to this view. A study of this mucoid material was therefore undertaken and the findings are now reported.

TECHNIQUE.

Strains employed.

Three mucoid strains of hæmolytic streptococci were used ; all belonged to Group A. Two of these strains, L. 193 and L. 212, were highly virulent for mice ; L. 166 was only of moderate virulence. L. 166 and L. 212 did not fall into any of Griffith's types ; L. 193 was a type 13 strain. In addition to these mucoid strains a non-mucoid form L. 275 was investigated. It belonged to Group A and was only of moderate virulence for mice ; it was not typed.

The strain of pneumococcus Type III—A 66—used in this investigation came originally from the Rockefeller Institute.

Production of sera.

These were made in rabbits, and the method employed was essentially the same as that described by the author for the production of protective sera against hæmolytic streptococci (Loewenthal, 1938). It depended on the use of suspensions of young encapsulated cocci killed by heating for 12 minutes

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at 55° C. While six intravenous injections at intervals of 4–5 days were usually sufficient to produce sera of high protective power, a longer course of immunization was necessary to obtain sera with a high titre for the polysaccharide.

Isolation of the polysaccharide from mucoid material.

From bacterial cells.—Of the earlier attempts to isolate and purify the mucoid material only one need be mentioned. The washings from large quantities (10-litre batches) of cultures in serum broth or serum neopeptone broth or bacterial extracts prepared by freezing and thawing in CO_2-snow or liquid air were subjected to fractional precipitation by alcohol at different hydrogen-ion concentrations. Further purification was obtained by removal of the proteins by trichloracetic acid followed by dialysis. A protein-free material was finally obtained, but the yield was small—only a few milligrammes from one litre of culture.

From culture fluid.—While work on these lines was in progress, a paper by Kendall, Heidelberger and Dawson (1937) on a serologically inactive polysaccharide elaborated by mucoid strains of streptococci appeared. Dr. Kendall most kindly supplied me with some of this material, and it was found to react in high dilutions with sera prepared against the encapsulated mucoid streptococci by the newly described method. It appeared, therefore, that the serological inactivity of the polysaccharide isolated by Kendall and his collaborators was due to lack of appropriate antibody in their sera and, this being so, their method of preparing the polysaccharide was tried out and finally adopted.

One litre of veal-peptone-phosphate broth containing 0·5 per cent. of glucose was inoculated with an ample inoculum of an actively growing culture of mucoid streptococci and incubated for 24 hours. The culture was then spun down in a Laval separator and the effluent sterilized by the addition of 0·5 per cent. of phenol. 100 g. of sodium acetate and 25 c.c. of glacial acetic acid were then added to it, and the polysaccharide precipitated with 1·25 volumes of alcohol. After deposition of the precipitate, which took about 48 hours, the supernatant was syphoned off and discarded. The precipitate was thrown down by centrifugation and dissolved in 200 c.c. of water. To this solution were added 10 g. of sodium acetate and 5 c.c. of glacial acetic acid, and it was then shaken mechanically with 50 c.c. of chloroform and 5 c.c. of butyl alcohol. Shaking with fresh portions of the solvents was repeated as long as a solid emulsion layer remained after centrifugation. The polysaccharides were then precipitated by 1·25 volumes of alcohol. The precipitate was dissolved in 100 c.c. of a 5 per cent. solution of sodium acetate containing 2·5 c.c. of glacial acetic acid and re-precipitated as before until the material was phosphate-free. Finally it was dissolved in 100 c.c. of water and precipitated by 500 c.c. of re-distilled alcohol; if necessary a few crystals of sodium acetate were added. After several washings with distilled alcohol the precipitate was filtered off and dried over calcium chloride. The yield from one litre of culture varied according to the amount of mucoid material produced by different strains: 80 mg. were obtained from strain L. 193 (Type 13).

Precipitin and absorption tests.

Precipitin tests.—The polysaccharide dissolved readily in water, but solution tended to become rather slow with higher concentrations. Precipitin tests were made by placing a layer of the antigen over the serum and noting the formation of a ring. The tubes were then placed in the incubator at 37° C. for 2 hours, being shaken after 30 minutes at this temperature; a reading was then taken and the racks were placed in the refrigerator overnight. A final reading was taken the next morning after the tubes had stood at room temperature for one hour. The tubes containing the lowest antigen concentrations were sometimes centrifuged to obtain a clearer reading.

Absorption tests.—The optimal proportions of serum and antigen were determined according to Dean and Webb's method, and a mixture of serum and polysaccharide solution prepared accordingly. The mixture was incubated for 2 hours at 37° C. and then left in the refrigerator overnight. After the precipitate had been removed by centrifugation, the supernatant was tested to make sure that the absorption was complete.

EXPERIMENTAL.

Serological Reactivity and Specificity of the Polysaccharide from Mucoid Material.

In the earlier stages of this investigation all attempts failed to demonstrate a precipitin reaction with the polysaccharide obtained from mucoid material. The antistreptococcal sera then used were prepared in the usual manner by immunizing rabbits with suspensions of streptococci grown for 18 hours and killed by either heat or formalin.

Meanwhile a method had been developed for the production of sera with high protective power against mucoid streptococci. This method entailed the use as antigens of suspensions of young capsulated cocci cautiously killed by heat (Loewenthal, 1938); with these sera the polysaccharide was precipitated in very high dilutions. Although it is recognized that this polysaccharide is probably not a complete antigen, for the sake of brevity it will be referred to here as the mucoid antigen.

While immunization with young capsulated cocci was found indispensable for the production both of precipitins for the mucoid antigen and protective antibody, the mucoid antigen was somewhat less readily destroyed than the type-specific antigen. Young cocci killed by formalin induced the formation of precipitins, and the mucoid antigen was found to be less sensitive to heat than the specific antigen, yet to obtain sera with high precipitin titres, young capsulated cocci killed by the minimal exposure to heat should be employed as vaccines.

When isolated and purified the mucoid antigen is relatively heat-stable; heating at 100° C. for half an hour does not destroy its serological activity. It can be prepared only from strains in the mucoid phase.

The results recorded in Table I show that the serum of a rabbit immunized with the mucoid streptococcus L. 193 not only precipitates the polysaccharide

TABLE I.—*Precipitin Tests with Serum of a Rabbit* (174) *Immunized with cautiously heat-killed young Cultures of a Group A Streptococcus L.* 193 *and the Polysaccharide isolated from various sources.*

Polysaccharide prepared from—		Least concentration of polysaccharide giving precipitin reaction.
Bacterium.	Colony form.	
Group A streptococcus (L. 193)	Mucoid . . .	1 in 2,000,000
,, ,, (L. 275)	Non-mucoid . .	<1 in 200.
,, ,, (L. 166)	Mucoid . . .	1 in 2,000,000.
,, ,, (L. 166)	Non-mucoid . .	<1 in 200.
Pneumococcus Type III		1 in 100,000.

prepared from the homologous strain to a very high titre, but also the preparation obtained from the heterologous strain L. 166 to an equal degree. This latter strain lent itself particularly well to a comparative investigation of different colony forms, since the mucoid (M) and the non-mucoid (N) forms bred true after they had dissociated, and could only be transformed by cultivation in immune sera. Neither the N form of strain L. 166 nor of strain L. 275 yielded any substance which reacted with this serum.

As can further be seen from Table I, a polysaccharide strongly reacting with antistreptococcal serum was also obtained from the culture fluid of a pneumococcus Type III.

That the mucoid antigen was not type-specific was further shown by tests made with the serum of a rabbit immunized with the Type 13 strain L. 193. The results of precipitin tests with this serum and the polysaccharide prepared from the homologous strain and an untypable strain L. 212, as well as with a preparation obtained by Dr. Kendall from a Type 3 strain, are recorded in Table II.

TABLE II.—*Precipitin Tests with the Serum of a Rabbit* (174) *Immunized with a Mucoid Streptococcus L.* 193 (*Type* 13) *and Polysaccharides prepared from Mucoid Streptococci belonging to different types.*

Polysaccharide prepared from—		Least concentration of polysaccharide giving precipitin reaction.
Strain.	Type.	
L. 193 .	13	1 in 2,000,000.
Kendall's .	3	1 in 2,000,000.
L. 212 .	Not typable	1 in 200,000.

It will be seen that the serum precipitated the polysaccharide from the strains belonging to the Types 13 and 3 equally well, and reacted, though less strongly, with the preparation made from the strain L. 212. Differences of a similar order were encountered with other sera, but the reason for their occurrence is not yet clear. The presence of other polysaccharides in the preparations might account for them, and this is probably the reason for the lower titres

obtained with the pneumococcal preparation, but the usual impurities, *i.e.* polysaccharides derived from agar and glycogen, are excluded by the method of preparation. In any case, these considerations do not impair the conclusion, drawn from the experiments in Tables I and II, that the mucoid antigen is not type-specific.

Relation between the Precipitin for the Mucoid Antigen and the Protective Antibody.

Is this serologically active polysaccharide connected with the invasive properties of streptococci, and is the presence of its corresponding antibody a necessary component of a protective antiserum ?

Protective antibodies are type-specific, whereas the mucoid antigen has been found to be common to different types and no parallelism between protective power and precipitin titre has been observed, so that it would seem that the answer to this question was in the negative. The matter was finally settled, however, by absorbing sera with the mucoid antigen, and testing their protective power in mice before and after removal of the precipitins.

The serum used for the purpose of this experiment was made by immunizing a rabbit with young-culture vaccine of a mucoid Group A strain L. 193 (Type 13). It precipitated the polysaccharide prepared by Dr. Kendall from a Type 3 strain as well as the preparation from the homologous strain in a concentration of 1 in 400,000 ; it possessed high protective potency. The optimum proportions of serum and polysaccharide were determined, and the serum absorbed by mixing with the appropriate quantity of antigen and placing the mixture for two hours at 37° C. and then overnight in the cold. A confirmatory test was made to ascertain that absorption was complete. Mice were injected with an amount of serum equivalent to 0·5 c.c. of undiluted serum, other mice receiving 0·5 c.c. of the unabsorbed serum. The control mice were given

TABLE III.—*Comparison of Protective Power of the unabsorbed Serum of Rabbit 207 Immunized with cautiously heat-killed young capsulated Cocci of strain L. 193 (Type 13), and of the same Serum after absorption with the Polysaccharide from Mucoid Material of a Type 3 strain.*

Dose of culture of L.193 in c.c. injected 18 hours after serum.	Mice receiving 0·5 c.c.							
	Normal serum.			Serum 207 unabsorbed.			Serum 207 absorbed with polysaccharide.	
10^{-2}	—	—	.	D1	D2	.	D2	D2
10^{-3}	—	—	.	D2	D2	.	D2	D3
10^{-4}	—	—	.	S	S	.	S	S
10^{-5}	D1	D2	.	S	S	.	S	S
10^{-6}	D2	D2	.	S	S	.	S	S
10^{-7}	D2	D3	.	S	S	.	S	S
10^{-8}	D2	D3	.	S	S	.	S	S
10^{-9}	S	S	.	—	—	.	—	—

— = Not tested. S = Survived. D = Died. Numerals indicate the number of days before the death of the animal after infection.

normal rabbit serum. As will be seen from Table III, complete removal of the precipitin for the mucoid antigen left the protective power of the serum unaltered.

DISCUSSION.

The experiments described prove that mucoid hæmolytic streptococci produce a substance of polysaccharide nature which streptococci in the non-mucoid phase do not form at all or only to a very small degree. This polysaccharide was found to be highly active serologically when appropriate sera had been prepared. Its extreme lability in the natural state is at first sight surprising, but the early controversy, with which the author was connected, concerning the antigenicity of the type-specific polysaccharide of pneumococci served as a reminder not to overlook the possibility of the denaturation of such an antigenic substance even by a treatment which at first sight might not appear detrimental.

The polysaccharide is not type-specific, and the antibody directed against it would not appear to play an important part in the protective power of an immune serum. The mucoid antigen thus seems unconnected with the invasive properties of streptococci, and the fact that an apparently identical substance can be isolated from a Type III pneumococcus shows that it is not peculiar to the mucoid material elaborated by the hæmolytic streptococcus. Indeed it is possible that it is widely distributed in nature, for according to Kendall, Heidelberger and Dawson (1937), and Meyer, Dubos and Smyth (1937), it may even be identical with a polysaccharide isolated by Meyer and Palmer (1936) from the vitreous humour of the ox and from Wharton's jelly in the human umbilical cord.

Apparently this polysaccharide is only fully antigenic when it is present in the young encapsulated coccus ; it has lost this power when it appears in the mucoid material. The secretion of the mucoid material is accompanied by the disappearance of the capsule, and it is conceivably the product of capsular breakdown. This would suggest that the polysaccharide is one of the capsular constituents, though not the important one as in the pneumococcus, since in the case of the streptococcus it has nothing to do with the production of protective antibody. Whatever may be the source of this polysaccharide or the significance of the secretion of the mucoid material, the finding of an antigenic substance peculiar to hæmolytic streptococci in the mucoid phase helps one to understand some part of the phenomenon of colonial variation exhibited by the streptococci. This question has been admirably reviewed by Hadley (1937), and lack of space forbids any detailed discussion of it here. Suffice it to say that the existence of the mucoid antigen explains fully the different qualities of the mucoid (M) and the non-mucoid (N) forms. It has been suggested that the difference between M and N forms was the presence in the former of an additional antigen which masks the type-specific antigen (Loewenthal, 1932, 1937), and a similar view was put forward by Dawson and Olmstead (1934). The mucoid antigen answers this description, and its lack of type-specificity accounts for Griffith's observation (1935) that the softer the colony is, the greater is the tendency to loss of type-specificity.

CONCLUSIONS.

1. A substance of polysaccharide nature can be isolated from hæmolytic streptococci in the mucoid phase and also from Type III pneumococci.

2. Its serological activity can only be demonstrated with sera produced with cautiously killed suspensions of young encapsulated cocci.

3. It is neither type- nor species-specific, and appears to be unconnected with the invasive properties of streptococci.

4. The fact that streptococci in the mucoid phase stimulate the formation of protective antibodies and precipitins for the polysaccharide from mucoid material only when in the young encapsulated state, can be explained by the process of deterioration of the bacterial surface which sets in after several hours of growth.

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